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Characterization And Function Of Iga In Hiv-1 Infection.

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CHARACTERIZATION AND FUNCTION OF IgA IN HIV-1 INFECTION

by

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A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title Characterization and Function of IgA in HIV-1 Infection

As the HIV-1 epidemic has continued to grow, development of an effective vaccine has become more imperative. Vaccination efforts are now being, in large part, directed toward eliciting protection at mucosal surfaces, where the IgA immunoglobulin isotype predominates. However, the function of both serum and secretory IgA in HIV infection is poorly understood. Questions have even arisen as to whether IgA might actually contribute to the pathogenesis of this disease. These studies were initiated to characterize certain aspects of serum IgA produced by HIV-infected individuals and determine what role, whether protective or pathogenic, IgA may play in HIV infection.

Concentrations of both the IgA1 and IgA2 subclasses were found to be proportionally elevated in the sera of the majority of HIV-infected individuals examined. Elevations in total IgA were determined to be mediated by increases in monomer; percentages of polymeric IgA1 and IgA2 were reduced. By western blotting it was discovered that despite increases in IgA2, only IgA1 recognizes HIV antigens, predominantly those of the envelope. After quantitating proportions of HIV antibodies in IgA and IgG, it was found that, in addition to nonviral IgA2, elevations of IgA1 also led to dilution of IgA HIV antibodies. Increases in concentrations of IgG, however, were accompanied by parallel increases in IgG HIV antibodies. These results suggest that within the systemic compartment of infected individuals, IgG B cell activation is antigen-driven, while much of the IgA1 and IgA2 B cell activation is polyclonal.

Low proportions of IgA antibodies reactive with a gp120 V3 loop peptide of North American consensus sequence could be detected in sera of infected individuals, suggesting type-specific neutralizing antibodies are generated. In addition, neutralization of HIV-1_{IIIB} by IgA could be achieved *in vitro*, despite a lack of significant reactivity with the HIV-1_{IIIB} V3 loop, indicating group-specific neutralizing IgA antibodies are also produced in response to HIV infection.

IgA and IgG purified from sera of 20 seropositive individuals were also examined for ability to enhance HIV-1 infection of the $Fc\gamma R^+$, $Fc\alpha R^+$ U937 cell line. As compared to IgG, twice as many individuals (70%) had IgA which enhanced infection of cells. Enhancement by IgA could be blocked if physiological concentrations of serum IgG were present. However, the ability of IgA to enhance HIV infection may contribute largely to the pathogenesis of this disease, particularly in the mucosa where much lower concentrations of IgG are present. In addition, these results suggest that enhancing epitopes on HIV recognized by IgA should be determined so that vaccines can be designed to induce IgA HIV neutralizing antibodies, rather than enhancing antibodies, at mucosal surfaces.

Abstract Approved by: Committee Chairman Susan Jackson

Program Director Susan Jackson

Date 12/31/94 Dean of Graduate School Joan F. Forden

DEDICATION

This work is dedicated to my incredibly supportive parents, George and Yvonne Kozlowski, who deserve highest honors for their understanding and tolerance of my rebellious nature, which began as a teenager and lasted well into college. Although raising me to be perseverant and independent, with a desire to explore, may have partly contributed toward their misery during those years, I must confess, guiltily, that I am grateful they instilled these qualities in me. Without them, I doubt I would have pursued a graduate education and discovered a career which promises to continue to interest and excite me for the rest of my life.

ACKNOWLEDGMENTS

I would like to acknowledge two individuals who have, over the years at UAB, unknowingly contributed a great deal to my enthusiasm for science and toward restoring my motivation during frustrating periods. All totaled, they have surely spent countless hours with me in helpful discussion and providing technical advice. To these individuals, Zina Moldoveanu and Mike Russell, I would like to express my deepest gratitude and best wishes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

INTRODUCTION

In 1981, the first clinical descriptions of a new immunodeficiency disorder, believed to be acquired through sexual contact, began to appear in medical peer review journals (Gottlieb et al., 1981; Siegal et al., 1981). The individuals described in these early reports were typically afflicted with Kaposi's sarcoma and plagued by opportunistic infections such as cytomegalovirus (CMV), *Candida albicans*, and *Pneumocystis carinii*. The virtual absence of peripheral blood $CD4^+$ T cells in these patients led to speculation that severe defects in cellular immunity, possibly initiated by an unknown viral infection, were responsible for their unrelenting infections (Siegal et al., 1981). Today, worldwide, the number of people infected with the retrovirus human immunodeficiency virus type 1 (HIV-1), responsible for the acquired immune deficiency syndrome (AIDS), is estimated at 14 million by the World Health Organization (WHO). In the absence of a vaccine and effective drug therapy, this number is expected to reach 30-40 million by the year 2000, with fatalities reaching catastrophic proportions due to the 100% mortality rate (WHO Working Group, 1994).

STRUCTURE AND LIFE CYCLE OF HIV-1

Both molecular and structural features of HIV-1 are similar to those of retroviruses in the lentiviridae family (reviewed by Peterlin & Luciw, 1988). However, in addition to the *env*, *gag*, and *pol* genes, other regulatory genes such as *tat*, *rev*, and *nef* make the HIV-1 genome one of the most complex among retroviruses (Fig. 1). Two copies of single-stranded genomic RNA, present in the nucleoid core of mature HIV virions, are associated with the reverse transcriptase (RT) enzyme and surrounded by a layer of the *gag* protein p24 (Fig. 1). Another protein layer formed by *gag*-encoded p17 is found immediately below the lipid bilayer of the viral envelope. The viral coat

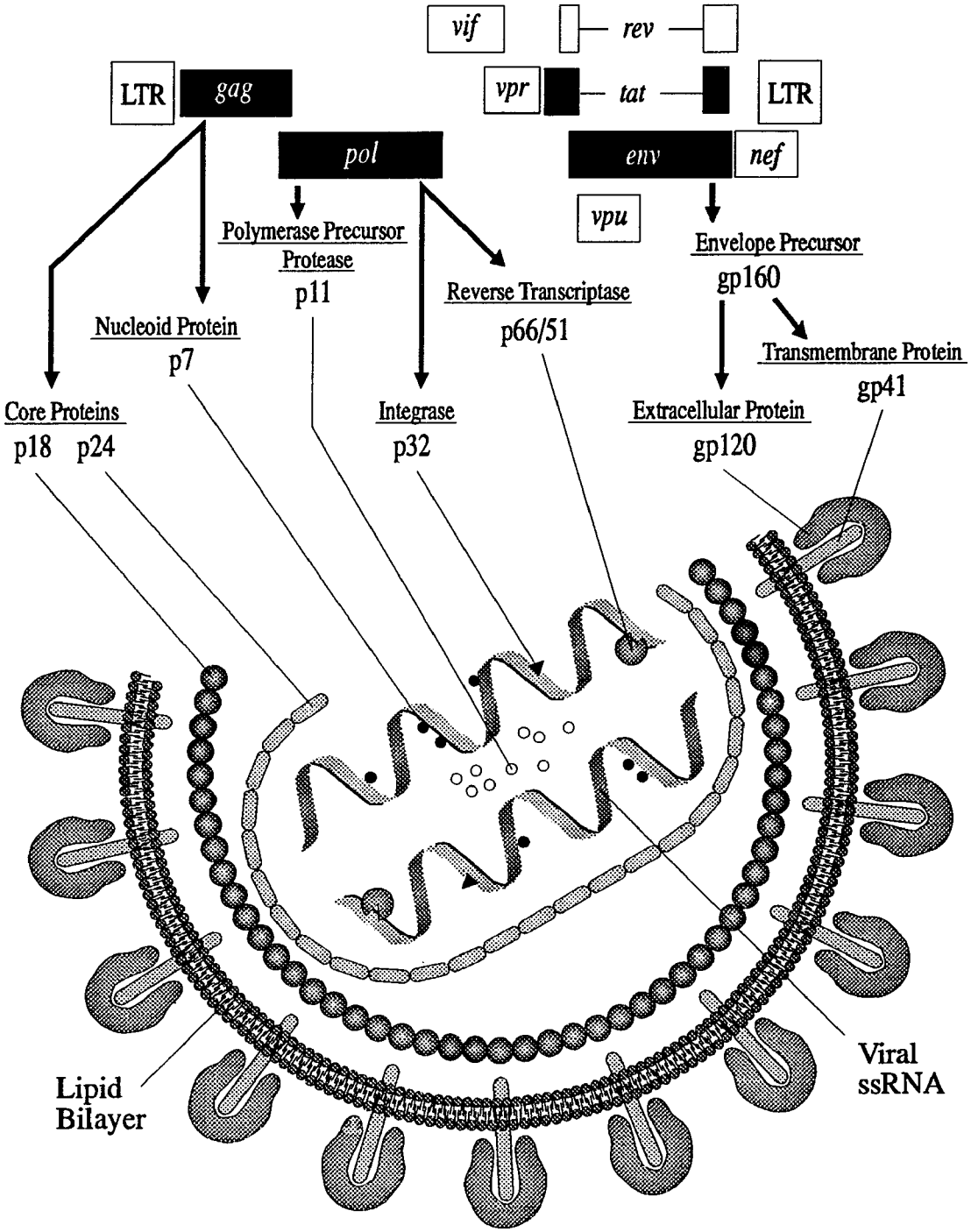

Fig. 1. Proteins encoded by the HIV-1 genome and structure of the mature HIV-1 virion. The coding regions of the HIV-1 genome are indicated, as are the major protein products.

displays 72 spikes of the *env*-derived noncovalently-linked glycoproteins gp120 and gp41. Other proteins such as MHC Class II antigens, derived from the host cell membrane, are also embedded in the lipid bilayer of the HIV envelope.

The predominant tropism of HIV for T cells and macrophages is a reflection of the high affinity interaction (4×10^{-9} M^{-1}) between gp120 and CD4 expressed on the surface of these cells (Lasky et al., 1987). The initial steps in HIV infection begin with binding of gp120 to CD4. In addition, it has recently been demonstrated that cell-surface expression of the CD26 serine protease is also necessary for viral entry and infection (Callebaut et al., 1993). It is now believed that following binding by the gp120 CD4-binding site to CD4, CD26 binds the third hypervariable region (V3) of gp120. Proteolytic cleavage of the conserved GP dipeptide sequence in the V3 domain by CD26 induces a conformational change which leads to dissociation of gp120 from gp41 (Callebaut et al., 1993). Evidence suggests that an amino-terminal hydrophobic region of gp41 then mediates fusion of the virion with the plasma membrane of the cell (Burney et al., 1988). However, the mechanisms involved in the fusion event are poorly understood; other regions of both gp120 and gp41 are also necessary for fusion to occur (Bolognesi, 1993). HIV can also enter cells in clathrin-coated vesicles which fuse with endosomes following receptor-mediated endocytosis (Pauza & Price, 1988; Grewe et al., 1990; Goto et al., 1988). As shown in Fig. 2, fusion of the lipid bilayer of the viral envelope with the lipid bilayer of the plasma (or endosomal) membrane allows the contents of the virion to spill into the cell cytosol where uncoating occurs, followed by reverse transcription of the viral RNA (Grewe et al., 1990; Bolognesi, 1990). The newly synthesized double-stranded viral DNA, containing two copies of the HIV long terminal repeat (LTR), is then translocated into the nucleus and integrated into the host genome.

Following cellular activation, host transcription factors such as nuclear factor-κB (NF-κB) bind *cis*-acting elements in the proviral LTR (Nabel & Baltimore, 1987) and lead to transcription of HIV genes, initiating the viral life cycle (Peterlin & Luciw, 1988).

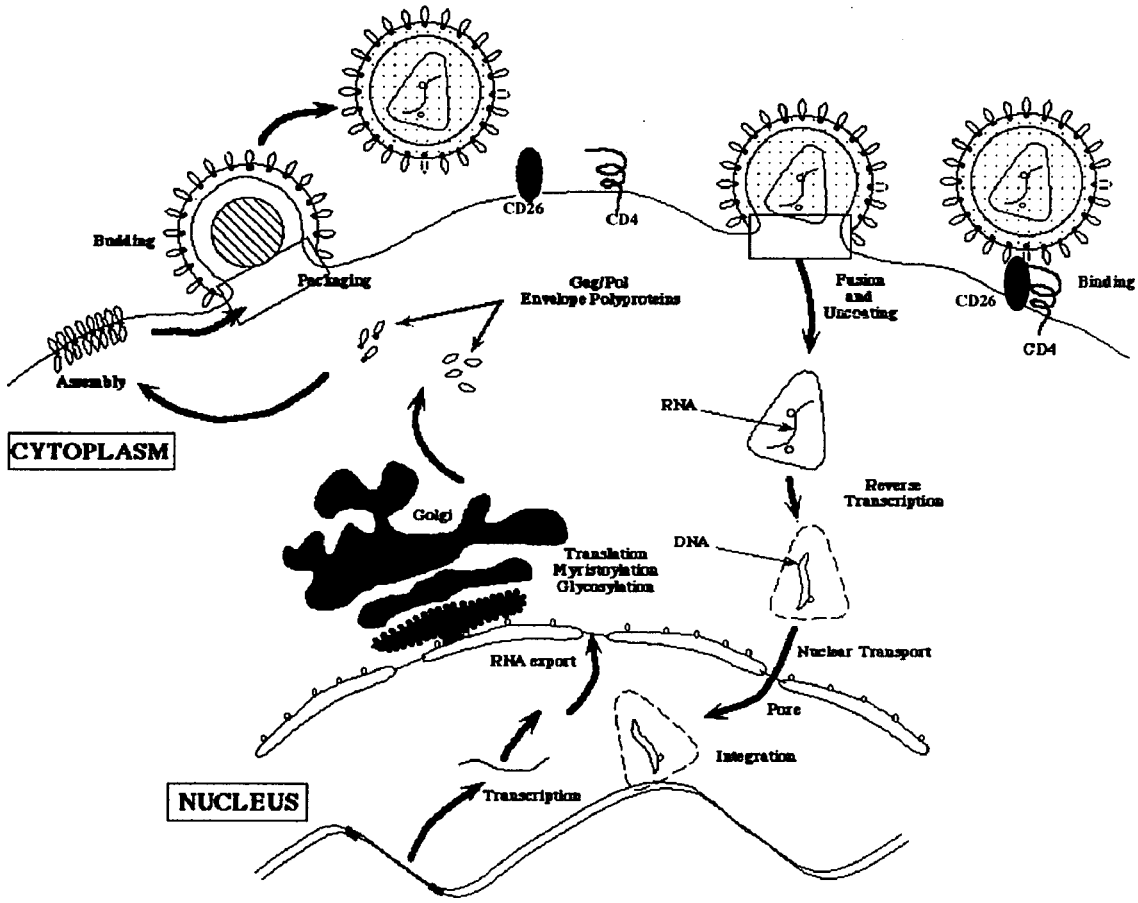

Fig. 2. The HIV-1 life cycle. As described in the text, the life cycle of HIV-1 begins with the binding of the virion to CD4 and CD26 cell-surface receptors. Fusion of the viral envelope with the plasma membrane of the cell allows the viral core to enter the cytoplasm where uncoating and reverse transcription of the single-stranded viral RNA to double-stranded viral DNA occurs. HIV-1 proviral DNA is then transported into the nucleus and integrated into the host DNA. Subsequent to transcription of viral DNA, translated HIV-1 proteins are assembled and packaged into virions at the plasma membrane of the cell. Mature progeny virions then bud from the cell and re-initiate the HIV-1 life cycle through binding to other $CD4^+$, $CD26^+$ cells. (modified from Stevenson et al., 1992)

Viral particles with two copies of genomic HIV RNA, *gag* and *pol* proteins are first assembled in the cytoplasm. Final maturation of virions occurs during the budding process at the cell surface where the lipid bilayer containing envelope glycoproteins is acquired (Peterlin & Luciw, 1988). HIV virions are then released. The entire life cycle of HIV can be completed in approximately 36 hours (Kim et al., 1988), although integrated provirus may remain latent for months or years, particularly in macrophages (Bednarik & Folks, 1992).

B CELL ABNORMALITIES ASSOCIATED WITH HIV-1 INFECTION

The discovery that HIV-1 infects cells expressing the CD4 antigen led to the early hypothesis that selective depletion of $CD4^+$ T helper lymphocytes, the cell subset centralto development of many immune responses, was responsible for the inability of infected individuals to effectively cope with opportunistic infections. However, with the development of the HIV-antibody test and subsequent identification of infected individuals who were symptomless, it became evident that many immunologic abnormalities measured *in vitro* could be detected in seropositive individuals with normal or relatively intact numbers of $CD4^+$ T cells. More specifically, aberrant B lymphocyte function prior to significant depletion of T helper cells suggested that HIV-1 infection may be characterized by both T and B cell dysfunction. The severe consequences of B cell derangement may be illustrated pathologically by the clinical observation that following Kaposi's sarcoma, the second most common malignancy associated with HIV infection early in the epidemic was the manifestation of B cell lymphoid tumors such as Burkitt's lymphoma (Zolla-Pazner, 1984).

Reduced Responses to Antigens and Mitogens. Abnormal B cell function in HIV-infected individuals was initially suggested by *in vitro* functional analyses using peripheral blood mononuclear cells (PBMC) from AIDS patients (Lane et al., 1983). Reduced proliferative responses to both the T cell-dependent and T cell-independent B cell mitogens, pokeweed (PWM), and *Staphylococcus aureus* Cowan Strain I (SAC),

respectively, were observed. This unresponsiveness could not be related to excessive T cell suppression or an inability of T cells to provide help but rather appeared to be the result of an intrinsic B cell defect, since depletion of T cells from these cultures did not lead to restored proliferation after exposure to SAC. Similarly, reductions in production of immunoglobulin (Ig) by PWM-treated PBMC could not be reversed after removal of the subject's T cells and replacement by T cells from seronegative healthy donors.

These studies have since been confirmed (Katz et al., 1986) and expanded to show that reduced proliferation and production of Ig after exposure to mitogens are also features characteristic of PBMC isolated from HIV-seropositve subjects who have been categorized as either asymptomatic or having persistent generalized lymphadenopathy, both clinical groups which display relatively intact numbers of circulating $CD4^+$ T cells (Rogers et al., 1989). Abnormal *in vivo* B cell responses by infected individuals have also been evidenced by poor or absent antibody responses following immunization with the protein antigens tetanus toxoid and keyhole limpet hemocyanin (KLH), as well as polysaccharide antigens of *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* (Lane et al., 1983; Ammann et al., 1984; Ambrosino et al., 1987; Ballet et al., 1987).

Circulating B Cells and Spontaneous Immunoglobulin Secretion. The above experiments documenting reductions in B cell responses could be explained if B cells were somehow rendered nonfunctional in HIV infection. However, although poor B cell responses are noted after exposure to mitogens, unstimulated PBMC from HIV-infected patients have been observed to spontaneously secrete Ig *in vitro* at concentrations generally 10-fold over those of seronegative controls (Lane et al., 1983; Katz et al., 1986; Rogers et al., 1989). This phenomenon may be related to increased numbers of terminally differentiated plasma cells within the circulating B cell pool since analyses of the subpopulations present in PBMC of seropositive individuals have demonstrated elevated percentages of those bearing a phenotype characteristic of activated (transferrin

receptor$^+$, large) B cells (Martínez-Maza et al., 1987). Further, elevations in spontaneous IgG and IgM production by PBMC of these subjects were found to correlate with increased numbers of these circulating activated B cells (Martínez-Maza et al., 1987). The presence of greater numbers of activated, spontaneous Ig-secreting cells in PBMC of HIV-infected individuals has also been demonstrated through limiting dilution analysis (Yarchoan et al., 1986).

Monocyte depletion or addition of anti-interleukin 6 (IL-6) antibody has been found to dramatically reduce levels of spontaneously produced Ig by PBMC of infected subjects (Amadori et al., 1991). Furthermore, addition of recombinant IL-6 (rIL-6) to monocyte-depleted PBMC has been shown to partially or completely restore spontaneous Ig secretion (Amadori et al., 1991; Delfraissy et al., 1992). It should be noted that the presence of as few as 3-5% monocytes in enriched B-cell preparations could sustain increased Ig production (Delfraissy et al., 1992). In view of the contribution of IL-6 in the differentiation of activated B cells to Ig-producing plasma cells (Hirano et al., 1985), along with the demonstration of increased IL-6 production by monocyte/macrophage cultures infected with HIV or those simply exposed to inactivated HIV preparations (Nakajima et al., 1989), a role of monocytes and IL-6 in mediating spontaneous Ig production by PBMC would not be entirely surprising.

Decreased percentages of resting B cells have also been observed in conjunction with the increased fraction of activated B cells in PBMC of HIV-infected individuals (Martínez-Maza et al., 1987). This finding has been confirmed by others (Reddy et al., 1991), who have also demonstrated that absolute numbers of circulating B cells are decreased, beginning with earliest stages of infection and becoming more pronounced as disease progresses. Possibly, decreases observed in numbers of total or resting B cells reflect a loss of mature or memory B cells bearing rearranged Ig genes of the heavy chain variable region 3 (V_H3) Ig gene family. An absence of these normally abundant cells from blood and lymph node mantle zone regions has been noted in HIV-infected

individuals (Berberian et al., 1991). For reasons unknown, significant increases in the fraction of circulating immature, pre-B cells have also been detected in individuals with end-stage disease (Martínez-Maza et al., 1987). Whether this may be a compensatory mechanism in response to the loss of other B cells, as has been suggested by the increased frequency of B cells found utilizing the V_H1 Ig gene family, is not clear (Berberian et al., 1991).

Overall, the manifestation of decreases in resting B cells with increases in both activated and spontaneous Ig-secreting B cells in PBMC of HIV-infected individuals suggests that the poor B cell responses observed in previous studies were not related to a functional incapacitation of these cells but instead were probably a consequence of their highly activated state *in vivo*, which would render them incapable of being stimulated further *in vitro* by mitogens or antigens (Zolla-Pazner, 1984). This, with the demonstration of PBMC spontaneously producing antibodies which react against an influenza A recall antigen (Yarchoan et al., 1986), indicative of memory B cell reactivation, has been taken to suggest that an intense polyclonal activation of B cells accompanies HIV infection.

Hypergammaglobulinemia. In addition to profound lymphopenia, two other striking immunologic features first associated with AIDS were the presence of hypergammaglobulinemia and elevated levels of circulating immune complexes (CIC) in the serum of patients (Gottlieb et al., 1981; Siegal et al., 1981). Analyses of larger study populations indicate that as many as 80-90% of AIDS patients may demonstrate elevations in at least one Ig isotype and that 67% may exhibit elevations in two Ig isotypes (Zolla-Pazner, 1984). B cell hyperactivity appears to be more pronounced among those committed to IgG and IgA production since these isotypes are most frequently detected at elevated levels in the serum of HIV-infected individuals. Interestingly, the factors which influence or mediate these increases appear to differ; increases in concentrations of IgA and IgG do not occur concurrently in individuals who

exhibit elevations in both of these isotypes, nor are they associated with the same stages of disease. Increased IgG is observed very early in HIV infection at the asymptomatic stage and remains consistently elevated throughout progression. Serum IgA concentrations, on the other hand, are generally normal in asymptomatic individuals, increase with disease progression, and are most pronounced by end-stage disease (AIDS). In view of this, it is not surprising that a significant association between declining CD4 count of individuals has been observed with increased IgA but not with increased IgG (Fling et al., 1988; Reimer et al., 1988; Mizuma, et al., 1987; Lucey et al., 1992).

Although one laboratory examining spontaneous secretion of IgD, IgG, and IgM by PBMC has reported a positive correlation with serum concentrations of these isotypes in infected individuals (Mizuma et al., 1988), the presence of increased numbers of plasma cells in the circulation probably does not alone significantly contribute to serum hypergammaglobulinemia in view of the low (ng/ml) concentrations spontaneously produced by these cells *in vitro*. Furthermore, circulating lymphocytes are represented by B cells which are derived from different lymphoid compartments and appear to be in the process of migrating to sites other than the bone marrow, the primary source of serum Ig (Underdown & Mestecky, 1994; Kutteh et al., 1982). Many of these peripheral B cells, when stimulated, do not exhibit phenotypic characteristics of those found in bone marrow (Mestecky & Russell, 1986). For instance, although distribution of the IgA subclasses among plasma cells in the bone marrow has been found to be ~88% IgA1 and ~12% IgA2, correlating with the proportion of these subclasses (82% IgA1, 18% IgA2) in sera (Skvaril & Morell, 1974), roughly equal numbers of cytoplasmic $IgA1^+$ and $IgA2^+$ B cells are detected in PWM-stimulated PBMC of normal subjects (Conley & Koopman, 1982). In addition, 90-95% of the total serum IgA is monomeric; IgA secreted in PWM-stimulated cultures, however, has been shown to be primarily dimeric in nature (Kutteh et al., 1980) and, as will be discussed later, appears to be synthesized by mucosally derived antigen-stimulated B cells in the process of migrating to other mucosal tissues. Thus, it is

unlikely that elevations in serum Ig concentrations can be solely related to the presence of increased numbers of circulating spontaneous Ig-secreting cells in HIV-infected individuals. The most probable source of hypergammaglobulinemic Ig is the bone marrow; in agreement with this hypothesis, increased numbers of plasma cells have been demonstrated in the bone marrow of seropositive patients classified as having AIDS-related complex (ARC) and AIDS (Sun et al., 1989).

Circulating Immune Complexes. Although, among the Ig isotypes, IgG hypergammaglobulinemia may be demonstrated in the serum of a higher percentage of seropositive individuals, the relative increase in this isotype averages 1.5-fold above normals and does not generally exceed elevations greater than 2-fold. In contrast, more dramatic elevations in IgA are observed, with average increases 2.5-fold above normals and elevations most often ranging from 2- to 7-fold (Fling et al., 1988; Reimer et al., 1988; Lucey et al., 1992). Elevations in these isotypes may contribute to the formation of both non-HIV- and HIV-containing CIC, implicated in the development of idiopathic thrombocytopenia purpura, vasculitis, and glomerulosclerosis that are often diagnosed in seropositive individuals (Euler et al., 1985). Significant associations between increased levels of CIC and increased concentrations of IgG in serum of AIDS patients have been demonstrated by some (Lin et al., 1988; McDougal et al., 1985), although not by others (Morrow et al., 1986). Interestingly, most pronounced elevations of CIC have been observed in the sera of patients with advanced disease, as have elevations in IgA, and negatively correlate with the percentage of $CD4^+$ T cells (Euler et al., 1985; McDougal et al., 1985).

Immunoglobulin isotype and antigenic content of CIC. It appears that early reports describing the isotypic composition of these immune complexes may have erroneously underestimated the content of IgA in CIC. Analysis of CIC in sera using methods based on the presence of or binding to the classical complement component C1q generally indicated increases in CIC containing IgG. However, these assays were biased

because, unlike IgG and IgM, the Fc region of IgA does not contain the C1q-binding motif, accounting for the inability of IgA to activate complement by the classical pathway (Russell & Mansa, 1989). Comparative analysis of sera for CIC using the C1q-binding assay and the Raji cell method, the latter being based on the binding of immune complexes to the Raji B lymphoid cell line via Fc, C3b, and C3d receptors, has clearly indicated this bias. With the Raji method, elevated CIC have been detected more frequently in serum of seropositive individuals and in many sera which were negative using the C1q-binding assay (Gupta & Licorish, 1985).

Increased detection, with the Raji assay, of CIC in sera of HIV-infected individuals probably reflects the presence of IgA immune complexes, which often contain cleavage products of the complement component C3 and may bind Raji cells through both complement and Fc receptors. In fact, a prevalence of IgA-containing CIC in serum of AIDS patients has been demonstrated using this assay (Lightfoote et al., 1985). In this study, CIC consisting of IgA alone could be detected, and although the presence of IgG in CIC was indicated, no CIC containing exclusively IgG could be found. This finding has been confirmed by others (Jackson et al., 1988a) and expanded to show that the IgA present in CIC is restricted to the IgA1 subclass. The reasons for this are not entirely clear but have been speculated to be related to the finding that elevations in serum IgA are mediated by increases in IgA1 but not IgA2 (Reimer et al., 1988).

The antigenic component of CIC detected in HIV-infected individuals has remained enigmatic. Although hepatitis B and *Pneumocystis carinii* antigens have been detected occasionally in CIC, no significant associations between immune complex levels and the presence of secondary infections or malignancies have been found (McDougal et al., 1985). Convincing data demonstrating the presence of either HIV or anti-HIV antibodies in these immune complexes have also not been provided. Thus, at this time there is no clear evidence to support a contention that elevated levels of immune complexes in the serum of HIV-infected individuals are formed in direct response to HIV.

Autoantibodies as mediators of CIC formation. Autoimmune phenomena such as the presence of anti-lymphocyte and anti-platelet antibodies in sera are commonly associated with HIV infection (Kopelman & Zolla-Pazner, 1988; Silvestris et al., 1989; Bettaieb et al., 1989). It is therefore possible that immune complex formation may be mediated through non-HIV antibodies. A high incidence of anti-IgG Fc-directed rheumatoid factor (RF) and elevated titers of other antiglobulins directed against IgG $F(ab')_2$ and the IgG heavy chain constant region 1 (C_H1) domain have been detected in serum of HIV-infected subjects (Jackson et al., 1988b; Sölder et al., 1989). Several observations suggest that antiglobulins may contribute to the formation of CIC. First, as with serum IgA concentrations, IgA RF is significantly raised in the serum of AIDS patients (Procaccia et al., 1987; Jackson et al., 1988b). Second, IgA RF has been demonstrated in CIC (Jackson et al., 1988b). Finally, IgA RF is also restricted to the IgA1 subclass as reported for IgA constituting CIC (Jackson et al., 1988a, 1988b). The presence of mixed IgG and IgA complexes would also be consistent with the formation of IgA anti-IgG immune complexes. However, the presence of CIC containing IgA exclusively supports the notion that the formation of these immune complexes is probably not mediated solely through IgA RF activity against IgG.

POLYCLONAL ACTIVATION OF B CELLS

It is clear that the earliest theories attributing all immune defects in HIV infection to the loss of $CD4^+$ T helper cells cannot rationalize many of the above abnormal humoral aspects, particularly, the manifestation of increases rather than decreases in Ig production and increased numbers of circulating activated B cells. There now appears to be general agreement among many investigators that most of the above-described B cell phenomena associated with HIV infection are characteristic of and can be attributed to hyperactivity within the B cell compartment resulting from an intense polyclonal activation of B cells. How this polyclonal activation may be mediated, however, has been difficult to conclusively establish and, not surprisingly, may be multifactored.

Transformation of B Cells by Epstein-Barr Virus. As with HIV infection, infectious mononucleosis induced by the herpes virus, Epstein-Barr virus (EBV), is also characterized by enhanced antibody production both *in vivo* and *in vitro*, inverted CD4/CD8 ratios, anergy, and decreased responses to PWM (Edelman & Zolla-Pazner, 1989). In addition, the EBV genome has been detected in B cell lymphomas arising in AIDS patients (Sonnabend et al., 1983). This has led investigators to consider the possibility that infection with HIV may result in polyclonal activation through reactivation of latent EBV infections with subsequent T-cell independent EBV-mediated transformation of B cells.

EBV-seropositivity among HIV-infected individuals is extremely prevalent, up to 100% in one study population, but active infection has been indicated in only one third of these individuals (Sonnabend et al., 1983; Rogers et al., 1983). Furthermore, although increased frequencies of spontaneously immortalized B cells have been observed in cultures of lymphocytes from ARC and AIDS patients (Yarchoan et al., 1986), others have found that spontaneous Ig-secreting B cells from HIV-infected individuals with hypergammaglobulinemia were not infected with EBV (Crawford et al., 1984). Although the possibility cannot be entirely excluded that B cell hyperactivity may arise partly as a result of EBV-mediated transformation in some individuals, it appears that it is not the sole mechanism responsible for these phenomena in others.

Direct Infection of B Cells with HIV. Although HIV has been shown to infect $CD4^-$ fibroblastoid, hepatoma, and glial cells (Werner et al., 1990; Cao et al., 1990; Kunsch et al., 1989), no evidence demonstrating productive infection of normal B cells by HIV has been provided. EBV-transformed B cells are susceptible to HIV infection, presumably as a result of EBV-mediated induction of surface CD4 expression (Tozzi et al., 1989), also supported by the demonstration of CD4 mRNA in these B cells (Spickett & Dalgleish, 1988). Although HIV infection of EBV-transformed B cells could contribute to pathogenesis through increasing proliferation and dissemination of HIV, it

seems unlikely that this phenomenon alone significantly influences polyclonal activation since, as mentioned above, active EBV infections are only detected in a minority of seropositive individuals and since a lack of correlation between EBV-infected and spontaneous Ig-secreting B cells has been demonstrated.

HIV Antigens as Mediators of Polyclonal Activation. Although normal B cells cannot be infected with HIV, it appears that antigenic components of HIV may somehow influence B cell responses. Purified normal B cells have been shown to proliferate in response to recombinant and synthetic peptides representing two highly conserved regions of the HIV envelope, one near the carboxy terminus of gp120 (amino acids 487-511) and the other in the immunodominant epitope (amino acids 578-608) of gp41 (Nair et al., 1988). In addition to increasing proliferation, elevated IgG and IgM secretion has also been observed in cultures of purified B cells briefly exposed to infectious HIV (Schnittman et al., 1986).

Inactivated preparations of HIV have also been shown to lead to increased Ig secretion in cultures of normal PBMC (Pahwa et al., 1985), but Ig production in response to PWM or SAC mitogens was inhibited if disrupted virus was added at the time cultures were initiated (Pahwa et al., 1985). This reduced responsiveness of normal PBMC to mitogens parallels that observed with lymphocytes isolated from infected individuals and further indicates that the inability to respond is not due entirely to the presence of infected T cells but may instead be related to an intrinsic property of an HIV protein.

B cell activation in relation to monocytes and T cells. Some controversy exists as to whether the stimulatory effects of HIV antigens may be directly exerted on B cells or mediated through either T cells or monocytes. HIV-induced Ig secretion in enriched B lymphocyte cultures has been shown to be dependent on the presence of T cells in one study (Yarchoan et al., 1986) but not in another (Schnittman et al., 1986). Both of these laboratories have reported that this HIV-mediated B cell activation was not dependent on the presence of monocytes. However, this conclusion may not be valid since these

cultures did contain contaminating monocytes and since, as mentioned earlier, others have found that complete removal of monocytes from purified B cell cultures abrogates HIV-induced Ig secretion (Amadori et al., 1991; Delfraissy et al., 1992). The presence of only 3-5% monocytes can quite effectively support Ig production initiated by live or inactivated HIV (Delfraissy et al., 1992). Discrepancies may also be in part related to the normal donor from whom cells were purified, the HIV isolate used, and even the cell line in which virus was passaged. The ability of different HIV isolates to induce IgG and IgM synthesis in normal B cell cultures has been found to vary greatly among donors (Spickett et al., 1989). This has also been evidenced in another study where inactivated HIV was able to induce Ig secretion by highly purified B cells (0% monocytes) from some donors but not others (Pahwa et al., 1986). Although the presence of T cells augmented increased Ig production by B cells in this latter study, it seems that in some individuals B cells alone can be directly stimulated by HIV.

Effect of HIV Antigens on Monocytes. Inactivated HIV and recombinant gp160 (rgp160) have also been shown to induce production of HIV-specific antibodies in cultures of monocytes and B cells from HIV-infected individuals (Delfraissy et al., 1992). In this study, the ability of HIV antigens to induce HIV antibody production was found to be related to the presence of monocytes since this effect was abrogated upon removal of these cells. Because the addition of IL-6 to monocyte-depleted cultures has been shown to completely restore spontaneous Ig production by PBMC (Amadori et al., 1991) and was able to partially restore antibody production by B cells, production of this lymphokine by monocytes or macrophages has been suggested to contribute to the activation of B cells. As mentioned earlier, inactivated HIV preparations have also been shown to induce IL-6 production in monocyte/macrophage cultures (Nakajima et al., 1989). Therefore, it appears that cells of this lineage may also be susceptible to dysregulation as a consequence of HIV-binding properties and that the abnormal production of soluble factors by these cells may also influence B cell responses.

Membrane TNF-α as a Mediator of Polyclonal Activation. Another potential mediator of B cell polyclonal activation may be tumor necrosis factor-α (TNF-α) expressed on the surface of HIV-infected T cells. Recently, it has been shown that although membrane TNF-α is not present on normal T cells, HIV-infected $CD4^+$ T cell clones express membrane TNF-α and actively secrete this cytokine in the absence of stimuli (Macchia et al., 1993). These T cell clones were capable of inducing proliferation and Ig secretion, particularly of IgA, when co-cultured with autologous B cells, an effect abrogated by the inclusion of antibodies specific for TNF-α or the TNF-α receptor. That $CD4^+$ T cells of HIV-infected individuals do express membrane TNF-α *in vivo* was demonstrated by FACS analysis. Further, T cells isolated from a lymph node specimen of an HIV-seropositive individual were also able to induce synthesis of IgA, IgG, and IgM by autologous B cells but not in the presence of anti-TNF-α receptor antibody.

This study may be noteworthy for several reasons. First, these authors specifically examined production of IgA by B cells, which has been overlooked in other studies. Second, in view of the often more extreme elevations of IgA in serum of infected individuals, it may be relevant that approximately 2.5-fold greater concentrations of IgA over IgG were secreted by lymph node B cells. In addition, the proportion of T cells expressing membrane TNF-α in HIV-infected individuals was inversely related to CD4 count, as has been found for serum IgA concentrations. Finally, HIV-infected monocytes and macrophages and noninfected but activated B cells, monocytes, and macrophages have all been reported to produce high levels of TNF-α and IL-6 (Beutler & Cerami, 1986; Rieckmann et al., 1990), which together may potentially augment activation and terminal differentiation of B cells in HIV-infected individuals.

Concentrations of IL-6 and TNF-α have also been found at increased levels in both PBMC cultures and sera of HIV-infected study subjects (Breen et al., 1990; Wright et al., 1988; Roux-Lombard et al., 1990). Most pronounced elevations in serum TNF-α levels have been observed in patients with end-stage disease (Lähdevirta et al., 1988;

Kobayashi et al., 1990; von Sydow et al., 1991), where elevations in serum IgA concentrations have also been found to be most dramatic. Whether soluble TNF-α may also participate in the activation of B cells committed to IgA synthesis has not been established. However, it is possible that the membrane form of this cytokine expressed on the surface of other B cells, monocytes, and macrophages could play a role in B cell activation, as does contact with membrane TNF-α^+ HIV-infected T cells.

It would be of interest to know whether inactivated preparations of HIV are also capable of inducing expression of TNF-α on the membranes of monocytes, macrophages, and T and B cells, as they have been found to induce IL-6 secretion by monocyte/macrophage cultures. This might partly explain the confusion regarding the necessity of these cells in the above studies where the effects of HIV on B cells were examined. Membrane TNF-α on normal T cells can be induced with phytohemagglutinin (PHA) or a combination of ionomycin and 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA), activators of protein kinase C (Macchia et al., 1993; Israël et al., 1989). Studies have shown that the binding of gp120 to T cells triggers the release of intracellular calcium and the inositol phosphate second messenger system, which leads to protein kinase C activation (Kornfeld et al., 1988). It is therefore not unreasonable to speculate that gp120 might induce membrane TNF-α expression on T cells and that the presence of these cells may be partially responsible for augmenting production of Ig by B cells where noted in the above studies. In addition, it remains to be determined whether membrane TNF-α on HIV-infected T cells in conjunction with IL-6 produced by infected macrophages may possibly together contribute more profoundly to the polyclonal B cell activation associated with HIV infection.

EVIDENCE FOR AN HIV ANTIGEN-DRIVEN HUMORAL IMMUNE RESPONSE

Although many B cell phenomena such as spontaneous Ig secretion and hypergammaglobulinemia are speculated to be the result of an intense polyclonal B cell activation in HIV-infected individuals, there is evidence which contradicts the

manifestation of a uniform polyclonal activation among all B cells. In addition, antigen-driven responses specifically generated against HIV have been demonstrated within the B cell compartment.

Absence of Polyclonal Antibody Against Recall Antigens. Increased detection of serum antibodies directed against a wide array of pathogens to which infected individuals have been previously exposed would be expected to accompany a nonspecific activation of B cells. It is true that a higher prevalence of antibodies to herpes viruses (EBV, CMV, simplex types 1 and 2), hepatitis A, and *Treponema pallidum* has been demonstrated in HIV-seropositive patients (Rogers et al., 1983). However, this appears to reflect the manifestation of active, ongoing infection by these sexually transmitted organisms in patients. Antibodies directed against many endemic pathogens are not detected at higher frequencies within the HIV-infected population (Rogers et al., 1983). In fact, a lower prevalence and titer of antibodies to some antigens has been found in HIV-seropositive sera (Rogers et al., 1983; Ammann et al., 1984; Yarchoan et al., 1986).

Others have examined the specificity of the Ig spontaneously produced by B cells and were not able to demonstrate that a significant reactivation of memory B cells accompanies HIV infection. Despite serological evidence indicating prior infection of HIV-infected individuals with hepatitis B or EBV, Ig spontaneously produced *in vitro* by PBMC from these individuals contained very few or no antibodies directed against these viruses (Amadori et al., 1988, 1989). One laboratory has reported that anti-A/Aichi (influenza A) antibodies could be detected in culture medium from unstimulated PBMC of seropositive but not seronegative subjects, but these antibodies were present in serum of the infected subjects at concentrations approximately 65% lower than those in serum of normals (Yarchoan et al., 1986).

HIV-Specific Antibody Responses. HIV IgG antibodies have been found to be produced by 20-40% of activated circulating B cells, correlating with the observation that 24-33% of the total Ig spontaneously secreted *in vitro* by PBMC reacts with HIV

(Amadori et al., 1988, 1989). This frequency of B cells secreting HIV antibodies is extremely high when compared to the 0.5-2% frequency of antigen-specific secreting B cells normally detected in seronegative individuals following immunization (Amadori et al., 1988) and may arise as a result of chronic stimulation by HIV. Others have reported that 1-3% of the total serum Ig in seropositive patients is HIV-specific (Yarchoan et al., 1986). It is conceivable that the discrepancy between proportions of HIV-specific antibodies produced by PBMC and those present in sera may be related to different characteristics of the microenvironments in various lymphoid compartments, as will be discussed later. Nevertheless, both of these studies do indicate that in HIV-infected individuals, a significant component of the B cell response is HIV directed.

HIV-Specific IgG Antibodies and Elevations in Total IgG. Although proportions of IgG HIV-specific antibodies present within total IgG in infected individuals have not been directly quantitated, elevations of serum IgG appear to be partly mediated by increases in HIV antibodies. Some support for this has come from the finding that hypergammaglobulinemic IgG is due specifically to increases in the IgG1 and IgG3 subclasses (Reimer et al., 1988), the same subclasses which have been found to dominate the IgG HIV-specific response in western blot analyses of sera from seropositive subjects (Chiodi et al., 1989; Sundqvist et al., 1986). That a large component of the IgG produced by infected individuals may be directed against HIV was initially suggested by the high incidence of monoclonal or oligoclonal Ig reported in the sera of these subjects (Papadopoulos et al., 1985). Others studying HIV-associated gammopathy have observed that most of these sera exhibit 2-3 different monoclonal Ig (Briault et al., 1988). Isotypic analysis indicates that the majority of oligoclonal Ig detected in these individuals is of the IgG1 subclass, followed by IgG3. Interestingly, with the exception of nine children with AIDS in whom IgA was the single monoclonal Ig detected, no monoclonal IgA could be detected in HIV-infected adults participating in this study (Briault et al., 1988).

The prevalence of IgG1 and IgG3 oligoclonal gammopathies in HIV infection suggests that the antigenic specificity of this Ig may be HIV related. Evidence supporting this hypothesis has come from studies in which sera were isoelectrofocused and subsequently transferred to nitrocellulose preblotted with HIV. Oligoclonal IgG present in sera was found to be directed mainly against HIV antigens (Amadori et al., 1990). This finding, taken together with the knowledge that elevated IgG, monoclonal IgG, and IgG HIV antibodies are all predominantly associated with the IgG1 and IgG3 subclasses, suggests that hypergammaglobulinemic IgG may not simply be nonspecific and polyclonal in nature but, rather, may be generated as the result of an HIV-driven response. More recently, molecular characterization of HIV-specific IgG antibodies from bone marrow of an HIV-infected asymptomatic individual (Barbas et al., 1993) and those produced by EBV-transformed B cells from other asymptomatics (Andris et al., 1991) has shown extensive somatic mutation of these antibodies, indicative of an antigen-driven response.

SHORTCOMINGS OF THE HUMORAL IMMUNE RESPONSE TO HIV

Despite the high frequencies of HIV-specific B cells and levels of IgG HIV antibodies present in seropositive individuals, it is obvious that this vigorous humoral response does not lead to elimination of virus from the infected host. The reasons for this appear to be partly related to a failure of the humoral system to keep pace with the high rate of HIV mutagenesis (Goodenow et al., 1989), particularly evident among the envelope glycoprotein genes (Hahn et al., 1985).

Inadequate Protection by Neutralizing Antibodies. HIV type-specific neutralizing antibodies have been shown to react against the principal neutralizing determinant (PND) contained within the V3 domain of gp120 (Javaherian et al., 1989). This region is essential for virus infectivity; most evidence indicates it may be involved in the fusion process (Bolognesi, 1993). The V3 domain is a disulfide-bonded loop structure containing a remarkably conserved GPG tripeptide at its crown but flanking amino acids

of extreme variability among isolates (LaRosa et al., 1990). Although antibodies produced against the V3 loop have been found to be most potent in neutralizing HIV, the hypervariability associated with the V3 domain renders most sera incapable of neutralizing at this site unless presented with autologous virus. Further, it is this region that has been found to undergo mutation under the selective pressure of specific antibody (McKeating et al., 1989; Nara & Goudsmit, 1990).

Broadly reacting group-specific neutralizing antibodies (capable of cross-reacting with different isolates) predominantly recognize the CD4-binding site on gp120 (Skinner et al., 1988) and appear later in the course of infection than do isolate-specific V3-directed antibodies (Bolognesi, 1991). Other less-characterized but potential HIV neutralizing epitopes have been reported to reside within the cytoplasmic portion of gp41 (Broliden et al., 1992) and both the first and second variable domains of gp120 (McKeating et al., 1993).

Although type-specific neutralizing antibodies can be demonstrated in individuals following seroconversion (Bolognesi, 1991), the presence of neutralizing antibody has been found to be ineffective at inhibiting cell-to-cell-transmission of HIV (Gupta et al., 1989). A selection for neutralization-resistant viral variants has further been shown to occur when HIV-infected cells are cultured in the presence of neutralizing antibody (McKeating et al., 1989; Robert-Guroff et al., 1986). The capacity of seropositive sera to neutralize autologous virus has also been found to decline as disease progresses and appears to be related to the emergence of these neutralization-resistant variants *in vivo* (von Gegerfelt et al., 1991). Others (Arendrup et al., 1992) have observed that generation of antibodies specific for escape mutants does eventually occur, but the observed delay in production of these neutralizing antibodies would permit further dissemination of virus. Therefore, it appears that a sluggish response on the part of B cells in the production of antibodies capable of virus neutralization may be partly responsible for the inability of the humoral system to effectively eliminate HIV.

Antibody-Dependent Enhancement of HIV Infection. Concerns have been raised in regard to the possibility that HIV antibodies produced by infected individuals or elicited through vaccination of seronegatives may exacerbate disease through an ability to enhance rather than neutralize HIV infection. Antibody-dependent enhancement (ADE) of viral infection was first described 30 years ago with Murray Valley encephalitis virus, West Nile virus, and Japanese encephalitis virus (Hawkes, 1964). Since this time, *in vitro* infection of Fc receptor$^+$ target cells by many other viruses has been shown to be capably enhanced in the presence of sub-neutralizing virus-specific antibody (Porterfield, 1986; Takeda & Ennis, 1990).

Pathogenesis of dengue viral infections in relation to ADE. The pathological consequences of enhancing antibody *in vivo* have been best illustrated following natural infection of humans with serotypes of the dengue flavivirus, endemic to Thailand, where development of dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) is associated with significant morbidity and mortality in children (Kliks, 1990). Although primary exposure to any dengue serotype is manifested as the benign dengue fever, the presence of pre-existing antibodies, often maternally derived, against one of the other dengue serotypes renders the individual at high risk to develop DHF or DSS following exposure to a different dengue serotype (Kliks, 1990).

Studies with this virus in rhesus monkeys have demonstrated that following infection with dengue virus type 1, 3, or 4, subsequent exposure to dengue virus type 2 leads to the development of higher levels of viremia in these animals (Halstead & O'Rourke, 1977). The ability of dengue-specific antibodies to enhance infection of moncytes and macrophages by this virus as much as 10- to 100-fold *in vitro* is now well established (Porterfield, 1986; Halstead, 1981). Further, enhancement has been shown to be specifically mediated via the binding of nonneutralizing dengue-specific IgG antibodies complexed with virus to Fcγ receptors on these cells (Littaua et al., 1990).

Cellular events associated with ADE of HIV infection. Antibodies specific for visna-maedi virus, a member of the lentivirus family which includes HIV-1, have also been shown to mediate enhanced infection of macrophages *in vitro* (Kennedy-Stoskopf & Narayan, 1986). The intracellular mechanisms which allow antibody-enhanced viruses to escape lysosomal degradation are not clear, but a potential mechanism which has been proposed will be discussed shortly.

Increased numbers of extracellular visna virions bound to cells and increased rates in internalization and uncoating of virus have been implicated in the augmented uptake of this virus in the presence of immune sera (Jolly et al., 1989). HIV-seropositive sera alone and seropositive sera in the presence of active complement have also been shown to enhance binding and subsequent internalization of HIV-1_{IIIB} and HIV-1_{Ba-L} in human monocyte cultures (Bakker et al., 1992) and increase binding of HIV-1_{RF} to and proviral formation in the MT-2 T cell line (June et al., 1991). Enhanced production of infectious HIV-1_{IIIB} by MT-2 in the presence of complement and seropositive sera has also been correlated with increased RT activity in culture medium and elevated levels of HIV-1 RNA in these cells (Robinson et al., 1989).

Diversity of HIV isolates and cell types participating in ADE. ADE of HIV infection differs from that of C-ADE in that the presence of HIV-specific antibodies in the absence of complement can lead to increased viral production. Sera or purified IgG from seropositive individuals have been shown to mediate ADE of infection in various cell types with many different HIV isolates: HIV-1_{IIIB} and LAV_{bru} in the monocytoid U937 cell line (Takeda et al., 1988; Takeda et al., 1990; Joualt et al., 1989; Zeira et al., 1990; Matsuda et al., 1989); HIV-1_{SF128A} in PHA-stimulated PBMC (Homsy et al., 1989); and HIV-1_{SF128A}, HIV-1_{Ba-L}, and HIV-1_{IIIB} in both peripheral blood monocytes and primary macrophage cultures (Homsy et al., 1989; Perno et al., 1990; Laurence et al., 1990; Takeda et al., 1990). In addition, as discussed below, sera have also been found to enhance infection by homotypic isolates in normal PBMC and macrophages (Homsy et

al., 1990). The finding that many different isolates can be enhanced in various cell types indicates this phenomenon is not just a property associated with the commonly used HIV-1_{IIIB} isolate or a particular target cell line.

Magnitude of ADE of HIV infection. Enhancement via HIV-specific antibodies in sera or IgG has generally been found to occur at nonneutralizing dilutions or serum dilution equivalents ranging from 10^{-3} to 10^{-6} and at a low multiplicity of infection (moi), from 0.01-0.0002. Although the magnitude of enhancement is usually much less than that observed with dengue virus, the 2- to 10-fold increases in HIV production observed could still play a significant role in pathogenesis through contributing *in vivo* to increased proliferation and expansion of virus during the prolonged course of HIV infection.

Frequency of enhancing antibodies in HIV-infected individuals. The prevalence of ADE among sera of HIV-infected individuals has been difficult to establish due to the small study groups examined. In addition, it may not be possible to accurately determine the prevalence of ADE among the HIV-infected population since this activity probably varies among sera in regard to many factors such as the antigenic specificity of antibodies, the HIV isolates used for study, and the receptors on target cells which may participate in ADE. ADE activity has been reported in 62% of seropositive sera (Zeira et al., 1990) and in 31% of purified IgG (Laurence et al., 1990) using U937 cells and HIV-1_{IIIB}. Those evaluating ADE in normal PHA-stimulated PBMC and primary macrophage cultures using sera and homotypic isolates have observed ADE of PBMC at a frequency of 7/16 (44%); of these seven, three also enhanced infection of their own HIV isolate in macrophages (Homsy et al., 1990). Importantly, the ADE activity reported here was primarily observed in the sera of patients with AIDS (5/16).

Involvement of CD4 and Fc receptors in ADE of HIV infection. With the exception of one laboratory (Homsy et al., 1989), ADE of HIV infection has consistently been shown by other groups to be dependent on the expression of CD4 by target cells. In addition, the ability of sera or IgG to mediate ADE has been found to be related to the

presence of FcγR receptors (FcγR) on the surface of cells. Both FcRI and FcRII IgG receptors have been implicated using U937 cells (Takeda et al., 1990; Laurence et al., 1990). Others have found that ADE of HIV infection by serum in primary macrophage cultures is mediated through the IgG FcRIII receptor (Homsy et al., 1989). These results suggest that all FcγR may actively participate in ADE. Although direct evidence has not been provided, ADE is believed to proceed via Fc receptor-mediated endocytosis of IgG-HIV complexes following the binding to FcγR (Takeda & Ennis, 1990).

One laboratory (Shadduck et al., 1991) has been unable to demonstrate significant ADE activity in the sera of 12 HIV-seropositive individuals using either HIV-$1_{Ba\text{-}L}$ or HIV-1_{IIIB} and primary monocyte/macrophage cultures. The inability of this laboratory to demonstrate enhancement may simply be the result of using a typical neutralization protocol, in which virus and antibody are allowed to remain in cultures for up to 5 days, instead of a standard enhancement assay, which involves short-term exposure to cells. The ability of seropositive sera to mediate ADE has clearly been demonstrated by many others; thus, the potential hazards which might arise following immunization of vaccine recipients should not be discounted.

Complement-Mediated ADE of HIV Infection. Heat-inactivated HIV-seropositive sera which do not enhance HIV infection of cells *in vitro* have been reported to do so when a source of active complement is included at the time infection is initiated (Robinson et al., 1988). This phenomenon, termed complement-mediated ADE (C-ADE), has been studied almost exclusively with HIV-1_{IIIB} using EBV-transformed B cell lines and the MT-2 or MT-4 T cell lines, all of which coexpress CD4 and CR2, the C3dg complement receptor (Tremblay et al., 1990; Gras & Dormont, 1991; Tóth et al., 1991). It has been shown that the presence of these receptors on cells and HIV-specific antibody and alternative pathway activation in sera are essential for demonstrating C-ADE activity (Robinson et al., 1988, 1990b; Tremblay et al., 1990; Gras & Dormont, 1991). The magnitude of C-ADE appears to be similar to that of ADE, ranging 2- to 10-fold over

controls. Whether C-ADE could contribute to the pathogenesis of HIV infection, particularly during EBV infection, at which time infected individuals could possess B cells susceptible to C-ADE, has not been determined.

C-ADE and interference with antibody-mediated neutralization. In addition to the possibility that increased production and dissemination of virus may be facilitated through combinations of complement and HIV antibodies, it has been suggested that the activity of neutralizing antibodies in sera may be diminished in the presence of complement. This has stemmed from the observation that a marked reduction of the HIV neutralizing titer in seropositive sera can be demonstrated in the presence of normal human sera having complement activity (Tóth et al., 1991: Robinson et al., 1988). Reduced neutralizing potential of sera in the presence of complement has been found to be more pronounced in later stages of disease, when the prevalence of C-ADE activity in sera is also highest (Tóth et al., 1991).

Potential Mechanisms for Enhancement of HIV Infection. Although the precise details involved in enhancement have not been elucidated, potential mechanisms have been theorized (Takeda & Ennis, 1990; Robinson et al., 1989) based on studies with other viruses, the CD4 receptor requirement, and intracellular events known to accompany receptor-mediated endocytosis. There is little doubt that the first stage of enhancement involves the binding of antibody and/or complement-opsonized HIV to Fc or complement receptors expressed on the cell surface. As mentioned above, the binding of complexed HIV to these receptors has been shown to lead to the concentration of more virus on cell surfaces than that observed with free virus binding to CD4 alone. Surface CD4 may augment the binding of these HIV complexes to the cell. For instance, if low-affinity antibodies mediate enhancement, then the high-affinity interaction between CD4 and gp120 could serve as an anchor for Fc-bound complexes. This interaction could contribute to internalization of complexes by more effectively triggering FcR-mediated endocytosis through increased cross-linking of receptors. However, although CD4

appears to be necessary in demonstrating ADE and C-ADE, it may not be involved in initial binding to the cell surface since binding of HIV to cell surface CR2 in the presence of complement and antibody can occur independently of CD4 (Montefiori et al., 1992).

As shown in Fig. 3, following endocytosis of Fc-bound antibody-complexed HIV virions in clathrin-coated pits, it is believed that coated vesicles containing the receptor-ligand complex fuse with prelysosomal vacuoles, termed endosomes, as characteristically observed with other receptor-ligand complexes (Marsh, 1984; Goldstein et al., 1985; Ukkonen et al., 1986). It is speculated that prior to reaching lysosomal vesicles, where degradation of contents would occur, the envelope of HIV fuses with the endosomal membrane such that the contents of the virion are emptied into the cytoplasm. This phenomenon has been observed with Semliki Forest virus and other enveloped viruses which utilize receptor-mediated endocytosis mechanisms to enter cells (Helenius et al., 1980; Marsh, 1984). In addition, electron microscopic examination of HIV entering cells via the endocytic pathway in the absence of antibody has demonstrated that virions first appear in clathrin-coated vesicles, then in vesicles partially devoid of clathrin, and finally in endosomes (Grewe et al., 1990). Direct fusion of the HIV envelope with the lipid bilayer of the endosomal membrane has been subsequently observed (Grewe et al., 1990). In the case of Fc-mediated entry of HIV virions complexed with antibody, CD4 may not be required until the virion is in the endosome, where a gp120-CD4 interaction with the endosomal membrane (derived from the cell-surface membrane) could be required to permit penetration into the cytoplasm.

The model developed for C-ADE is similar to that above but incorporates the requirement for both alternative pathway activation and CD4 (Robinson et al., 1989). Antibodies capable of activating the classical pathway of complement must first bind the HIV envelope. Following classical pathway activation, various complement components, including C3, are deposited on the surface of the virion. It is not until C3 fixation on HIV is amplified through the alternative pathway, however, that binding to the CR2 receptor is

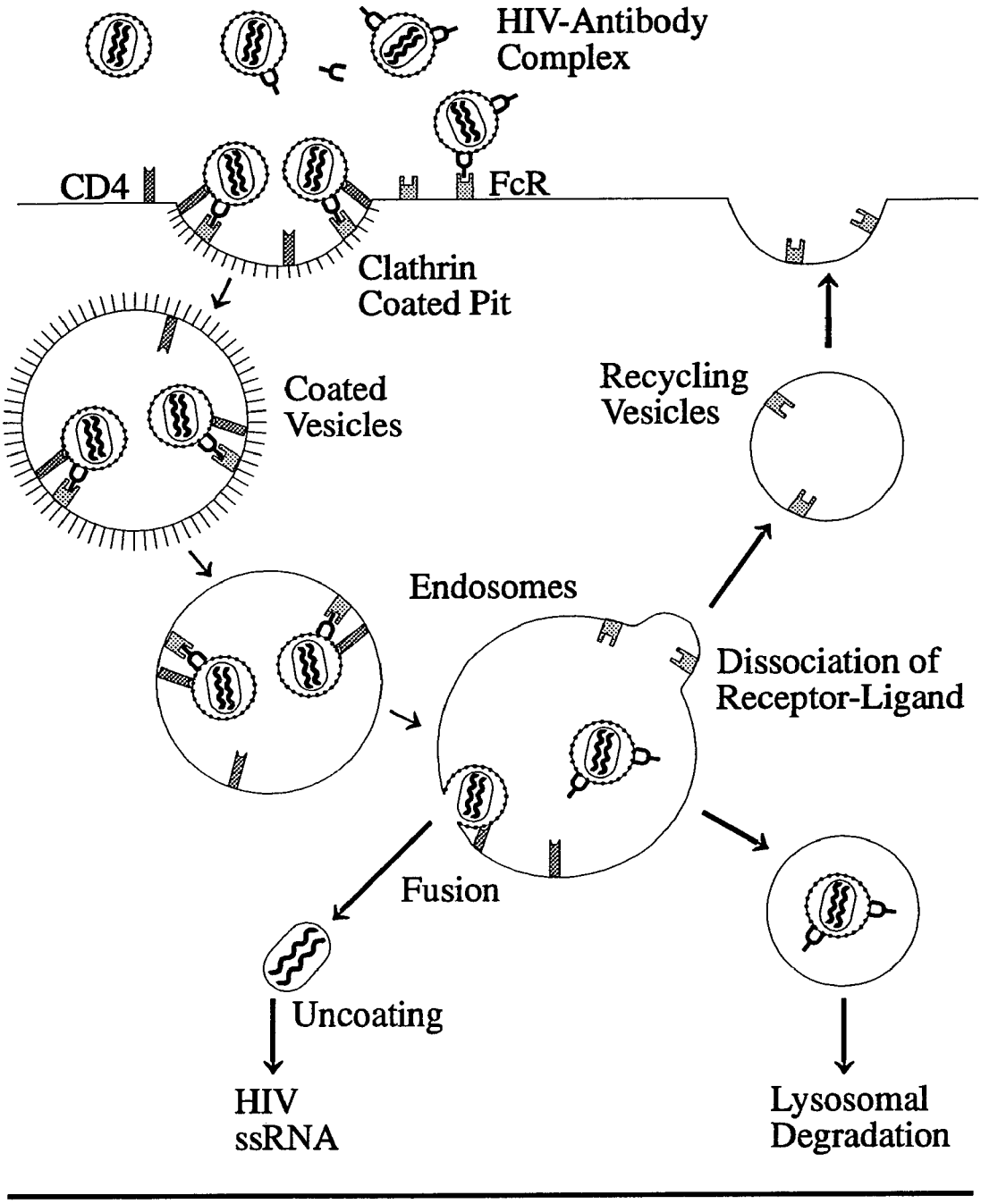

Fig. 3. Proposed model for entry of antibody-complexed HIV into cells via FcR.

proposed to occur. It has been speculated that some HIV antibodies once bound to virus may actually directly activate the alternative pathway, but there is no direct evidence to support this hypothesis. This model states that in addition to virus binding CD4, more HIV virions may be localized on the FcR^- MT-2 or MT-4 cell surface through interaction with the CR2 receptor. As opposed to the binding affinity of each receptor alone for virus, it is hypothesized that a higher affinity interaction of complement-coated HIV with the cell may be achieved through coassociation of virions with both CR2 and CD4, leading to enhanced internalization and the intracellular events described above.

HIV Epitopes that Mediate Enhancement. HIV epitopes which may elicit antibodies that mediate enhancement have been reported to reside within both gp120 and gp41. A human IgG1 monoclonal antibody (mAb), designated 2F11, produced by EBV-transformed tonsillar B cells from an asymptomatic individual and found to react with $gp160_{SF-2}$ and both gp41 and gp160 of HIV-1_{SF33}, has been shown to enhance HIV-1_{SF128A} but not HIV-1_{SF-2} or HIV-1_{SF33} infection of PBMC (Eaton et al., 1994). By peptide mapping, 2F11 has been shown to recognize a region spanning amino acids 579-599 of gp41 (Eaton et al., 1994). Three other human mAb (one IgG2, two IgG1) which mediate enhancement of HIV-1_{IIIB} infection in MT-2 cells in the presence of complement have also been found to react against a highly conserved, immunodominant gp41 epitope contained within amino acids 579-613 (Gnann et al., 1987; Robinson et al., 1991). A fourth human mAb (IgG2), designated 120-16, has C-ADE activity but binds another gp41 domain, spanning residues 644-663 (Robinson et al., 1991), which also includes a less conserved immunodominant sequence (Gnann et al., 1987). A combination of 120-16 with monoclonals directed against the gp41 579-613 epitope has been shown to act synergistically in mediating C-ADE infection of MT-2 cells with HIV-1_{IIIB} (Robinson et al., 1990a, 1991). This is somewhat alarming since antibodies in sera of HIV-infected individuals react more frequently with a highly immunogenic sequence (584-609)

contained within the gp41 579-613 amino acid region than with any other HIV determinant (Neurath et al., 1990; Shafferman et al, 1989).

Although the immunodominant gp41 579-613 epitope implicated in enhancement is highly conserved among HIV isolates, some evidence has been presented which suggests that enhancement could be mediated by antibodies that cross-react poorly with dominant HIV epitopes. One group has generated rabbit antisera against peptides corresponding to V3 loop sequences of different HIV isolates (Jiang et al., 1991). Antisera were then tested for their ability to mediate enhancement of HIV-1_{IIIB} infection in MT-2 cells in the presence of human complement. Although some antisera (11/21) such as that generated against the HIV-1_{IIIB} subclone HIV-1_{BH-10} could completely or partially neutralize infection, others (10/21) were found to enhance. Importantly, the ability of antisera to enhance infection was observed to significantly correlate with decreased crossreactivity against the HIV-1_{IIIB} V3 loop (Jiang et al., 1991).

Whether enhancement of HIV infection is mediated by antibodies generated against conserved or variable determinants of HIV, both are disturbing possibilities which should be investigated in more detail since this phenomenon could profoundly hinder efforts to develop a safe and effective vaccine for HIV-1. The discouraging results of recent vaccine trials (Cohen, 1993) emphasize the necessity for further study in this area. Although the inability to protect some vaccinees could be related to a failure to induce neutralizing antibodies which are capable of preventing infection by primary field isolates, the possibility cannot be excluded that vaccine failure may be related to the development of antibodies in vaccine recipients which are capable of enhancing infection of certain HIV isolates following transmission. Thus, it will be imperative to examine these antibodies in regard to both their potential to mediate ADE or C-ADE of HIV infection and their specificity so that enhancing epitopes of HIV can be eliminated from future vaccines.

IgA IN HIV INFECTION

Attempts to discern functional abnormalities within IgA B cell populations in HIV-infected individuals have been frustrated by a lack of examination of this isotype in many analyses. For instance, although IgG, IgM, IgD, and IgG HIV-specific antibodies have been shown to be spontaneously secreted by PBMC, spontaneous secretion of IgA or IgA HIV antibodies has not been examined. This neglect may have arisen partly from early erroneous reports that IgA anti-HIV antibodies were not present in the serum of infected individuals and partly from a lack of interest in IgA, the function of which in serum is poorly understood. That B cell abnormalities extend to this isotype in HIV infection has been convincingly indicated by the presence of elevations in serum IgA, IgA RF, and levels of CIC predominantly containing IgA in the serum of seropositive patients. There are also indications that HIV infection may lead to aberrant production of IgA associated with mucosal tissues.

Overview of IgA in the Systemic and Mucosal Compartments. Normally, the systemic and mucosal immune compartments operate relatively independently of one another. This can be partly illustrated by differences in the molecular and physiochemical characteristics of IgA associated with these areas. As previously mentioned, IgA in serum is produced by plasma cells in the bone marrow, 90-95% is monomeric, and most (80-85%) is distributed within the IgA1 subclass. Secretory IgA (S-IgA), on the other hand, is derived almost exclusively from plasma cells locally producing polymeric IgA (pIgA) and is distributed more equally among the IgA1 and IgA2 subclasses (McGhee & Mestecky, 1992). The division between the intravascular and mucosal compartments has also been illustrated in several studies which have shown that IgA produced in each of these compartments does not contribute to IgA levels present in the other: extremely low concentrations of S-IgA (~10μg/ml) are detected in serum (Underdown & Mestecky, 1994), and very little (<2%) of the IgA present in secretions is plasma derived (Delacroix et al., 1982; Jonard et al., 1984).

The polymeric Ig receptor and S-IgA. Following local synthesis of joining (J) chain-containing pIgA by plasma cells in subepithelial lamina propria or interstitial regions of mucosal tissues such as those in the salivary glands and gastrointestinal or respiratory tracts, IgA is transported into the lumenal mucous layer by serous-type secretory epithelial cells which express the polymeric Ig receptor (pIgR) on their basolateral surfaces (Brandtzaeg et al., 1994). The binding of pIgA to the pIgR leads to endocytosis and formation of a disulfide bond between the C_H2 domain of one α heavy chain and the fifth domain of the pIgR (Brandtzaeg et al., 1994). The receptor/ligand complex is transcytosed from the basolateral to the apical membrane, where proteolytic cleavage between the pIgR transmembrane region and the extracellullar fifth domain leads to release, into the lumen, of IgA. The five extracellular domains of this receptor which remain associated with such secreted pIgA are collectively known as secretory component (SC) (Underdown & Mestecky, 1994). The selective transport of locally synthesized pIgA from subepithelial mucosal regions, termed "effector sites," into secretions is similarly mediated by the pIgR in the nasal mucosa, mammary glands, and both the female and male reproductive tracts (Mestecky & McGhee, 1987).

Functions of S-IgA. The addition of SC to IgA is a feature which distinguishes S-IgA from serum IgA. The presence of SC in S-IgA also confers biological properties that further differentiate IgA in serum from that in secretions. S-IgA is less susceptible to proteolysis by both metabolic and microbial enzymes (Kilian & Russell, 1994). The protective functions of S-IgA have been well documented (Kilian et al., 1988; Kilian & Russell, 1994). Many of these activities are believed to be potentiated by the natural affinity of S-IgA for mucins. S-IgA is an extremely efficient agglutinator and has been widely credited for protecting the mucosal surfaces from colonization by invading viruses, bacteria, fungi, and other pathogens through an ability to neutralize viruses, toxins, and enzymes; inhibit attachment of bacteria to epithelial cells; and augment

destruction of bacteria and fungi by synergizing with nonspecific defense factors such as lactoferrin, lactoperoxidase, and lysozyme (Kilian et al., 1988).

Locally produced pIgA, which is also a powerful agglutinator, may contribute to the elimination of pathogens which successfully penetrate the epithelial barrier through opsonizing these microorganisms for phagocytosis by FcαR-expressing macrophages present in mucosal tissues (Kerr, 1990; Shen, 1992). Antigens may also be removed from these regions and directly deposited into the mucous layer following complexing by specific pIgA antibodies and translocation across epithelial cells expressing the pIgR (Kaetzel et al., 1991). In addition, there is some evidence that uncomplexed but antigen-specific pIgA undergoing pIgR-mediated transcytosis may have the potential to neutralize viral infections in epithelial cells (Manzanec et al., 1992).

Function of serum IgA. The biological functions of serum IgA are very poorly understood in comparison to S-IgA. IgA antibodies in serum have been found to react with a wide array of viruses. Serum IgA antibodies reactive against CMV (Linde et al., 1983), influenza A (Brown et al., 1985a), EBV (Yao et al., 1991), rubella (Mitchell et al., 1992), coxsackie B (del Rosario Zuniga et al., 1993), hepatitis delta (Chaggar et al., 1991), and respiratory synctitial (Meddens et al., 1990) viruses have been demonstrated in individuals afflicted with these viral infections. Although the biological significance of these IgA virus-specific antibodies in serum is still unknown, serum IgA could potentially contribute to the neutralization of viral infections.

S-IgA and serum IgA are incapable of activating the classical pathway of complement. Although controversial, IgA is also generally considered to be a very poor, if at all, activator of the alternative pathway (Russell & Mansa, 1989). Thus, IgA antibodies do not function in complement-mediated immune responses as do IgM or IgG antibodies. Much of the early work with IgA indicated that IgA in serum was poorly opsonic, did not mediate antibody-dependent cellular cytotoxicity (ADCC), and actually inhibited chemotaxis of neutrophils and activation of both complement pathways

(Griffiss, 1983; Shen, 1992). It has been speculated that IgA in serum may function as a regulatory, anti-inflammatory Ig because it blocks complement-mediated effector mechanisms which might be damaging to the host if left unchecked (Griffiss, 1983).

Serum IgA has been shown by some to participate with monocytes in ADCC-mediated killing of meningococci and phagocytosis of *Neisseria gonorrhoeae* (Lowell et al., 1980; Bisno et al., 1975). In addition, results of earlier studies have been challenged over the last few years as others have presented data contrary to these findings (Shen, 1992). There is some evidence which suggests that serum IgA could play a more active role in host defense than previously thought. For instance, it has recently been found that resistance to infection by *Schistosomiasis mansoni* in African children is associated with the presence of serum IgA antibodies specific for this parasite (Auriault et al., 1990). These serum IgA antibodies have been shown to react against the *S. mansoni* glutathione-S-transferase enzyme and exhibit potent neutralizing activity against development of more schistosome larvae by interfering with reproductive life cyle stages (Grzych et al., 1993).

Although myelomonocytic cell lines and peripheral blood monocytes have been found to phagocytose IgA-coated erythrocytes or latex beads (Maliszewski et al., 1985; Shen et al., 1986; Shen et al., 1989; Fanger et al., 1983), dimeric or aggregated IgA preparations were used in these studies, as in those demonstrating IgA can trigger superoxide production by peripheral blood monocytes and neutrophils (Shen, 1992; Hostoffer et al., 1993). Therefore, it is unclear whether monomeric IgA (mIgA) in serum may actually mediate these FcαR-related functions.

As with S-IgA, mIgA1 and mIgA2 do bind the FcαR (Monteiro et al., 1990), although weakly (Shen et al., 1989), with an affinity similar to that of the FcRII affinity for IgG (Kilian & Russell, 1994). Enhanced binding of pIgA as a result of increasing numbers of FcαR has been shown to occur following treatment of myelomonocytic cell lines with differentiation-inducing reagents (Monteiro et al., 1990; Shen et al., 1989;

Shen et al., 1986). Increased expression of the FcαR can also be induced on the surface of monocytes following incubation with bacterial endotoxin, TNF-α, IL-1, granulocyte/macrophage-colony stimulating factor (GM-CSF), and lipopolysaccharide (LPS) (Shen, 1992; Shen et al., 1994). Neutrophil FcαR may also be up-regulated by chemoattractants (Hostoffer et al., 1993). Increases in FcαR expression have been correlated with increased phagocytosis of IgA-coated targets by the HL-60 cell line (Shen et al., 1986). Enhanced binding of pIgA due to an increase in the affinity but not in the numbers of FcαR has also been observed to occur immediately following exposure of U937 cells to pIgA (Monteiro et al., 1990; Monteiro et al., 1992). Treatment of peripheral blood neutrophils with GM-CSF similarly induces a change from low to high affinity binding of IgA by FcαR, a change which leads to an ability of these cells to ingest IgA-coated latex beads (Weisbart et al., 1988).

It is conceivable that the high levels of antigen-specific pIgA antibodies which have often been detected in sera of individuals shortly after systemic immunization or development of viral or bacterial infections (Brown et al., 1985b; Lue et al., 1988; Tarkowski et al., 1990) could actively function with FcαR in the removal of antigens or pathogens from the circulation. However, this possibility and the question of whether up-regulation or increased affinity of FcαR for IgA may facilitate phagocytosis of mIgA-coated antigens have not been directly examined. Thus, the physiological relevance of the FcαR in regard to serum IgA function remains unclear.

Induction of antigen-specific S-IgA responses. Antigen-specific serum IgA but often not S-IgA responses can be induced through systemic immunization of individuals (Mestecky, 1986; Lue et al., 1988; Tarkowski et al., 1990). Site-restricted antigen-specific S-IgA antibodies can be elicited by direct application of antigens to various mucosal membranes or secretory glands (Mestecky & McGhee, 1987). However, synthesis of S-IgA antibodies at effector sites can also be induced through antigenic stimulation at more distant mucosal sites (Mestecky & McGhee, 1987). Much of the IgA

in secretions appears to arise as a result of the migration of antigen-stimulated IgA-committed B cells from organized lymphoid follicles, termed "inductive sites," present in the gut-associated lymphoid tissue (GALT) and bronchus-associated lymphoid tissue (BALT), to lamina propria or interstitial regions of these and other mucosal tissues where they terminally differentiate into plasma cells (Mestecky & McGhee, 1987).

In the small intestine, inductive sites containing mucosal plasma cell precursors are primarily associated with large aggregates of lymphoid follicles known as Peyer's patches (PP) (Mestecky & McGhee, 1987). Smaller lymphoid aggregates resembling PP are distributed throughout the respiratory tract and the large intestine, particularly the colon and rectum (Sminia et al., 1989; Mestecky & Jackson, 1994). The lumenal surfaces of these follicular structures and PP are characterized by their domed-shaped appearance, absence of villi, and a layer of $pIgR^-$ absorptive epithelium; interdispersed within are antigen-transporting microfold (M) cells, a novel epithelial cell found only at these sites (Kraehenbuhl & Neutra, 1992). The basolateral membrane of the M cell is characteristically invaginated to an extent that it is only several microns away from the apical membrane, forming a large intraepithelial space which is occupied by a group of lymphocytes (both T and B) and macrophages (Kraehenbuhl & Neutra, 1992). Immediately below this layer of epithelial cells is a large population of macrophages and T and B cells, residing intermediate between the epithelium and the organized lymphoid follicular region, which may contain several B cell germinal centers and surrounding parafollicular T cell zones (McGhee et al., 1989). In addition to macrophages, antigen-presentation in these lymphoid aggregates may be facilitated by dendritic cells present in both B and T cell areas (Mestecky & McGhee, 1987)

Mucosal immune responses are induced following the adherence of pathogenic viruses, bacteria, or other antigens to the apical M cell membrane. Internalization and transcytosis leads to delivery of antigen to intraepithelial lymphocytes and macrophages (Kraehenbuhl & Neutra, 1992). Immune responses subsequently generated result in the

stimulation of immature predominantly pre-committed IgA B cells, which exit the follicular regions via efferent lymphatics, migrating through the mesenteric lymph nodes to the thoracic duct where they enter the peripheral circulation. These B cells eventually enter lamina propria or interstitial regions of various mucosal tissues and glands where they terminally differentiate into plasma cells synthesizing antigen-specific polymeric IgA antibodies for transport into the secretions.

Possible HIV-Related Mucosal B Cell Aberrancies. Because many circulating B lymphocytes may be those that are mucosally derived and en route to effector sites, it is quite possible that a significant component of the spontaneous Ig-secreting cells observed in PBMC from HIV-infected individuals originates from mucosal sites. This could explain the observation that despite the presence of reduced concentrations of influenza A antibodies in sera, PBMC spontaneously produce these antibodies *in vitro* (Yarchoan et al., 1986). For reasons unknown, different patterns of reactivity against HIV have also been observed between antibodies in serum and those spontaneously secreted by PBMC of some HIV-infected individuals (Amadori et al., 1988). Although broad reactivity against all HIV antigens was noted in serum, restricted specificity for envelope glycoproteins was found by IgG present in PBMC cultures. The lower proportion of total HIV antibodies present in serum (1-3%) as compared to those secreted *in vitro* by PBMC (24-33%) may also be related to the presence of migrating mucosal B cells in PBMC cultures. However, 20-40% of the Ig spontaneously secreted by PBMC has been reported to be represented by IgG HIV antibodies (Amadori et al., 1989). Therefore, if PBMC do contain mucosal B cells spontaneously secreting Ig, this could indicate that not only are these cells abnormally activated in the circulation but development of IgA B cells may also be affected since those precommitted to IgG usually constitute a minority of the immature B cells present at mucosal inductive sites. A loss of normal regulation, as in the systemic compartment, is quite likely since the intestinal mucosa of HIV-infected individuals contains decreased numbers of CD4 T cells and increased numbers of CD8 T

cells (Smith, 1994). Furthermore, it is clear that HIV infects the mucosa (Smith, 1994). Infection by opportunistic pathogens such as CMV may also result in inflammation and abnormal regulation and lead to breaches in the barrier between the systemic and mucosal compartments. The finding that, in contrast to serum, IgA2 concentrations in saliva and parotid fluid of AIDS patients are significantly decreased (Jackson, 1990; Müller et al., 1991) suggests that another B cell disorder associated with HIV infection may be manifested as reduced function or numbers of mucosal IgA plasma cells.

IgA HIV Antibodies. The question of whether IgA HIV-specific antibodies are produced in infected individuals and may have a protective function became increasingly more important with concerns that infectious HIV may be transmitted through mucosal secretions such as saliva (Groopman et al., 1984), cervicovaginal fluid (Vogt et al., 1987), and breast milk (Thiry et al., 1985), where the predominant Ig isotype present is IgA. Consequent isotypic analyses of seropositive sera for HIV antibodies yielded contrary results in regard to the presence and specificity of IgA HIV antibodies. Although serum IgA antibodies specific for HIV *gag* proteins have been demonstrated in one study (Khalife et al., 1988) and for both *gag* and *pol* antigens in another (McDougal et al., 1987), IgA HIV antibodies could not be detected by ELISA in the sera of any HIV-infected individuals participating in another study (Mergener et al., 1987). The reason for these discrepancies is likely due to the lack of sensitivity and specificity of these assays for examining IgA responses in the presence of much higher concentrations of serum IgG antibodies. In addition to the possibility that IgA RF may react with HIV-specific IgG and lead to a false-positive result, IgG may also block the binding of HIV-specific IgA to viral antigens, producing false-negative results. This has been indirectly suggested in above studies and others (Sundqvist et al., 1986; Chiodi et al., 1989) by the observation that the IgG1 subclass, present at highest concentrations in sera, dominates the HIV response, particularly the anti-envelope response, which could not be demonstrated for IgA.

Utilizing a more sensitive method, in which IgA antibodies in seropositive sera were first bound to Protein A or acrylamide beads with a rabbit anti-human IgA antibody and then used to precipitate radiolabeled HIV antigens, it has been found that, in contrast to the previous studies, IgA in serum reacts predominantly with the envelope glycoproteins gp160, gp120, and gp41 (Archibald et al., 1987a), with less frequent detection of antibodies reacting with the gag protein, p24. Using this method, a similar pattern of reactivity against HIV has also been observed for IgA in parotid saliva and cervical secretions, suggesting HIV-specific IgA is present in secretions associated with both the oral mucosa and mucosa of the female reproductive tract (Archibald et al., 1987a, 1987b).

IgA HIV antibodies have since been detected by others in breast milk, cervical fluid, and vaginal secretions of seropositive women (Bélec et al., 1989a, 1990; Lu et al., 1993) and in semen of infected men (Bélec et al., 1989b). In these studies the pattern of reactivity reported for IgA in secretions must be interpreted with caution since a very high incidence of control seronegative IgG or IgA reacting with all HIV antigens, except those of the envelope, is often seen in both sera and secretions examined by this laboratory. This suggests that the demonstration of IgA antibodies which frequently recognize envelope glycoproteins in these secretions may be valid but not those against other antigens, particularly p24. Overall, in regard to a potential for IgA-mediated neutralization, it is encouraging that anti-envelope IgA antibodies are produced and can be demonstrated in serum and secretions. On the other hand, these antibodies could contribute to the pathogenesis of HIV infection if they have enhancing properties similar to those found with IgG.

It should be pointed out that the above experiments demonstrating HIV-specific IgA antibodies in serum and secretions did not examine these antibodies in regard to molecular properties associated with IgA such as monomeric versus polymeric forms, subclass distributions, or the presence of SC. Therefore, the nature and origin of HIV

IgA antibodies in both serum and secretions have not been fully elucidated. The possibility that IgA HIV antibodies may be passively transudated from serum into secretions cannot be ruled out.

SUMMARY AND AIMS OF THE CURRENT STUDY

It is clear that profound dysfunction among B cells occurs in the HIV-infected individual. Further, the aberrant production of IgA, evidenced by elevations in serum IgA, indicates that dysregulation extends to B cells committed to production of this isotype. However, very few of the studies documenting B cell disorders have included analysis of the IgA isotype. The relative contribution of this isotype in HIV infection has thus remained elusive. This body of work was designed specifically to characterize and examine the functional aspects of serum IgA in infected individuals. Because an ultimate goal of this laboratory involves determining whether neutralization or enhancement of HIV may be mediated by IgA at mucosal sites, it is hoped that the methodology developed and described herein can be extrapolated to similarly analyze functions of S-IgA. The knowledge obtained from such studies should also prove invaluable in evaluating efficacy of potential vaccines designed to specifically induce IgA-mediated protection at mucosal surfaces.

In the first paper of this dissertation, IgA in sera of infected individuals was specifically examined in regard to concentrations, proportions, and molecular forms of IgA1 and IgA2 in an attempt to discern the origin of elevated serum IgA. Although elevations likely arise from the bone marrow, the possibility that elevated IgA in serum is passively derived from mucosal tissues and transudates into serum as a result of a breach in the barrier between the mucosal and systemic compartments cannot be excluded. Because of the differences in the subclass distributions and molecular forms of IgA present in these compartments, analysis of the IgA present in sera should help elucidate the nature of these elevations. IgA was also purified from sera of infected individuals, and both subclasses were subsequently analyzed for reactivity against HIV proteins. This

analysis was performed for several reasons. First, the use of purified IgA should avoid interference problems associated with IgG and conclusively determine the antigenic specificity of this isotype. Second, differences between reactivity of IgA1 and IgA2 for HIV may exist, as indicated by the finding that only IgA1 is elevated in sera, is present in CIC, and has RF activity. Finally, future analyses regarding function are based on the premise that IgA will bind HIV antigens, studies which would be futile in the absence of IgA HIV antibodies.

In the second paper of this thesis, levels of IgA and IgG HIV antibodies, present in seropositive subjects with normal or elevated serum concentrations of these isotypes, were quantitated in an effort to determine whether elevated IgA or IgG may be mediated by antigen-driven increases in production of HIV-specific antibodies or instead may lead to dilution of HIV antibodies as a result of a generalized nonspecific polyclonal activation. In addition, recognition of a gp120 consensus V3 loop peptide by serum IgA was quantitatively analyzed to determine if IgA has the potential to function in the neutralization of HIV through binding to the PND. Binding of IgA to the $gp120_{IIIB}$ V3 loop was also studied and compared with the ability of IgA to inhibit HIV-1_{IIIB} infection of a T cell line *in vitro*. This latter analysis was partly performed to determine if IgA may mediate neutralization of HIV infection via binding to envelope determinants other than the V3 loop. The development here of an assay to evaluate IgA-mediated neutralization in serum could also be useful in future studies examining neutralization of HIV by S-IgA.

The third paper of this thesis specifically examines the potential for IgA-mediated enhancement of HIV via the FcαR using the U937 cell line. It is obvious that the consequences of enhancing phenomena *in vivo* could be devastating. Although the ability of IgA to enhance infection has not been directly examined, it is conceivable that enhancement activity previously demonstrated in seropositive sera may have been partly mediated by IgA. On the other hand, it is also possible that an ability of IgA to enhance HIV infection might be blocked by the presence of greater concentrations of neutralizing

IgG antibodies in serum. In this case, even if such an effect by IgA is masked in sera, a similar capability for enhancement of HIV infection by S-IgA could have profound pathological consequences at mucosal sites, where very little IgG is locally produced. It will therefore be imperative to further determine whether S-IgA may enhance HIV infection, particularly since many laboratories are now in the process of developing vaccines designed specifically to elicit protective IgA HIV antibodies in mucosal secretions. U937 is one of the few available cell lines which expresses the FcαR. The development of an assay to detect IgA enhancing antibodies using this cell line could therefore be very useful in subsequent analyses of S-IgA enhancing properties involving this receptor. In addition, this work may facilitate design of future assays with other cells to determine whether enhancement could occur through other IgA receptors such as the ASGPR expressed on hepatocytes.

Finally, IgA-mediated enhancement of HIV infection could also potentially occur through the pIgR expressed on mucosal epithelium. Several epithelial cell lines which express this receptor could be utilized for *in vitro* study of this phenomenon. We have discovered that one of these cell lines expresses particularly high levels of pIgR and spontaneously polarizes in culture, suggesting it might be an ideal candidate for studies of enhancement phenomena. The characteristics of this cell, in regard to those associated with other polarized epithelial cells, are included herein and the potential for use of this cell line in studies with IgA is discussed.

SERUM IgA SUBCLASSES AND MOLECULAR FORMS IN HIV INFECTION: SELECTIVE INCREASES IN MONOMER AND APPARENT RESTRICTION OF THE ANTIBODY RESPONSE TO IgA1 ANTIBODIES MAINLY DIRECTED AT *ENV* GLYCOPROTEINS

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Abstract

In a study population representing different CDC stages of HIV infection, 58% exhibited IgA hyperglobulinemia resulting from proportional increases in both the IgA1 and the IgA2 subclasses. These increases were detected early in infection, did not correlate with CD4 count, and remained elevated throughout disease progression. Absolute concentrations of polymeric IgA present within each subclass were unchanged, indicating that increased production of monomeric IgA1 and IgA2 were responsible for elevations of total IgA. These elevations were not completely attributable to a specific antibody response to viral infection, since Western blot analysis of purified IgA samples indicated that HIV-reactive IgA could be demonstrated only within the IgA1 subclass. Dominating IgA1 anti-HIV responses were also observed in 2 secretory IgA samples isolated from colostrum of healthy HIV-seropositive mothers, suggesting that a similar isotype restriction exists in the mucosal IgA compartment. The binding of IgA1 to HIV proteins contrasted markedly to that observed with identical concentrations of IgG purified from the sera of the same patients. While IgG reacted more intensely and more broadly with all HIV proteins, IgA1 antibodies were directed predominantly against envelope glycoproteins. In many patients, a total lack of IgA1 reactivity to *gag* and *pol* proteins was accompanied by intact IgG responses to these same antigens. Though all IgA samples examined reacted with HIV, fewer responses to gp160, gp120, and p24 were observed in samples from AIDS and AIDS-related complex (ARC) patients, suggesting a declining titer of IgA antibodies against these antigens may be associated with disease progression. However, the preference of IgA1 antibodies for HIV *env* proteins suggests that a potential role for IgA-mediated neutralization of HIV may exist in vivo.

Introduction

Immunoglobulin isotype responses to human immunodeficiency virus (HIV) have been characterized in a number of studies, most of which have described differences in IgG subclasses.[1-3] These data are of interest because of the known functional differences in

immunoglobulin isotypes and subclasses.[4] However, little information is available with respect to IgA anti-HIV responses, especially regarding the subclasses and molecular forms of IgA. The IgA system is unique in that systemic and secretory (mucosal) compartments exhibit a considerable degree of independence.[5] In humans, the majority (>90%) of mucosally derived secretory IgA (S-IgA) is comprised of polymeric, J chain-containing immunoglobulin. In serum, however, monomer dominates, with only 10-15% of the total IgA represented by polymer. Similarly, serum and S-IgA display disparate relative subclass concentrations: serum IgA usually contains about 85% IgA1, while external secretions generally contain no more than 60% of this subclass. Although several reports have described S-IgA anti-HIV antibodies in virtually all external secretions (for review, see ref. 6), little attention has been given to the potential role of serum IgA antibodies in HIV infection. This is somewhat curious in light of the often extremely elevated levels of IgA in infected individuals and the fact that IgA antibodies might interfere with elimination of virus through competition with complement-fixing IgG and IgM antibodies.[7-9]

Aberrancies of humoral immunity, including polyclonal B-cell activation and hypergammaglobulinemia, are a prominent feature of the acquired immune deficiency syndrome.[10-12] Although elevated concentrations of IgG are generally present in HIV-infected individuals, striking increases in serum immunoglobulins have also been found within the IgA isotype.[12-17] It is unlikely that these elevations in serum IgA are due to increased production of IgA in the gastrointestinal tract since the mucosal and serum IgA systems are relatively independent of one another and the origin of serum immunoglobulin, including IgA, is primarily the bone marrow.[5] Clearly, a better understanding and examination of the role of IgA in the pathogenesis of HIV infection is needed, particularly in regard to vaccines targeted toward eliciting mucosal IgA responses. In this report we characterize the IgA subclass and molecular form of total serum IgA and IgA anti-HIV antibodies throughout the various stages of HIV infection.

Materials and Methods

Subjects

Serum samples from HIV antibody-positive individuals were obtained from the UAB Center for AIDS Research Clinical Core Facility and stored at -70°C until use. The study population consisted of 80 patients, each clinically diagnosed as belonging to one of the following groups: Stage II (asymptomatic, ASY), Stage III (lymphadenopathy, LAD), Stage IVC-2 (AIDS-related complex, ARC), and Stage IVC-1 (AIDS). Each group was comprised of 20 patients. In addition, 20 healthy seronegative heterosexuals (10 males, 10 females) and 10 HIV antibody-negative male homosexuals voluntarily contributed serum to represent control groups. These latter groups are designated HTX and HMX, respectively. The mean ± SD age of these latter two groups were 32.9 ± 8.9 and 32.1 ± 9.8.

Each group consisting of HIV-infected volunteers contained 16 males and 4 females, except for the ARC group which was represented entirely by male patients. The mean ± SD ages of these groups, in order of disease progression, were 32.2 ± 8.0, 34.0 ± 10.2, 34.7 ± 7.9, and 35.5 ± 6.2 years. Mean ± SD CD4 counts, in same order, were as follows: 296 ± 245, 298 ± 225, 201 ± 286, and 32 ± 60 cells/mm^3. Respectively, within each of the 4 groups, 7, 12, 9, and 11 patients were receiving azidothymidine (AZT) therapy.

Immunochemical reagents

Murine monoclonal anti-human IgA1 and IgA2 antibodies (kindly provided by Drs. J. Radl and J. J. Haaijman, TNO Institute of Experimental Gerontology, Rijswijk, the Netherlands) were used as coating reagents in the ELISA assay. The sensitivity and specificity of these reagents has been verified.[18] These antibodies were biotinylated by previously described procedures for use in Western blot analysis.[19] Biotinylated polyclonal rabbit anti-human J chain antibody was the gift of Dr. J. Mestecky (UAB, Birmingham, AL). Affinity-purified, biotinylated $F(ab')_2$ fragments of goat anti-human IgA and anti-human IgG were purchased from TAGO (Burlingame, CA). Monoclonal anti-human IgA

(specific for IgA1 and IgA2) was prepared in this laboratory and its specificity verified by ELISA on cognate and non-cognate isotypes.

Purification of IgG and IgA

IgG was affinity-purified from serum on Protein G Sepharose 4 Fast Flow (Pharmacia, Piscataway, NJ). Serum IgA from 21 HIV-infected volunteers was purified by lectin column chromatography with agarose-bound jacalin (Vector, Burlingame, CA), which preferentially binds IgA1, or by affinity chromatography on a column of insolubilized monoclonal antibody specific for the Fc region of both IgA subclasses. This reagent was found to bind IgA1 and IgA2 at proportions equivalent to those present in serum (unpublished observations). IgA2 was isolated by passing affinity-purified IgA over jacalin and collecting unadsorbed fractions.

Western blotting

Samples of 300 μg of purified IgG or IgA in phosphate-buffered saline (PBS), pH 7.4, containing 3% bovine serum albumin (BSA) and 1% Tween-20 were incubated with HTLV-III_{RF} preblotted nitrocellulose strips (Universal Biotechnology, Rockville, MD) at 25°C for 12 h. Strips were then washed and treated with consecutive incubations in biotinylated antibodies for human IgG, IgA, IgA1, or IgA2, extravidin alkaline phosphatase conjugate (Sigma, St. Louis, MO), and NBT/BCIP alkaline phosphatase substrate buffer (Bio-Rad, Richmond, CA). In the production of these blots, virus is fractionated by electrophoresis on 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gels of 10 cm length. This short gel length leads to insufficient resolution of gp160 and gp120 and sample reactivity observed at this position is therefore denoted gp160/120.

ELISA

Levels of IgA1, IgA2, polymeric IgA1, and polymeric IgA2 were determined by quantitative ELISA as previously described.[18] Briefly, diluted sera were incubated on 96 well polyvinyl chloride microtiter plates (Dynatech, Chantilly, VA) coated with monoclonal antibodies specific for either IgA1 or IgA2. Plates designated for determination of

polymeric IgA within these subclasses were subjected to consecutive 30 min treatments of 0.2% glutaraldehyde and 5M urea. After washing, these plates were then incubated with biotinylated anti-J chain for 2 h, while plates designated for analysis of total IgA1 or total IgA2 were incubated with biotinylated anti-IgA. Subsequently, all plates were treated with alkaline phosphatase-conjugated strepavidin (Jackson ImmunoResearch, West Grove, PA), developed with enzyme substrate, and optical densities recorded at 405 nm in an automated plate reader (Anthos Reader 2001, BioQuip, Atlanta, GA). Total IgA was determined by combining IgA1 and IgA2 concentrations.

Statistics

Differences between groups were determined by one-way analysis of variance using Tukey's multiple comparisons procedure.

Results

Levels of serum IgA1 and IgA2 throughout disease progression

In a cohort of 80 patients representing various stages of disease, the levels of both IgA1 and IgA2 were elevated (Fig. 1). Further, these increases were proportional; no aberrations in the ratio of IgA1 to IgA2 were found when compared with noninfected individuals. Elevated levels of IgA1 in the sera of HIV-infected individuals were noted as early as ASY and were found to remain above normal throughout disease progression. Although IgA1 levels showed little variation within the seronegative control and within the seropositive groups, statistically significant differences were observed between both control groups and ASY, LAD, and AIDS patients. Mean IgA1 concentrations were greater in ARC patients than that of the control groups; however, this value was lower than those observed in the other seropositive groups, and was not significantly different from either control group. A summary of statistical differences between groups for total IgA, IgA1, and IgA2 is presented in Table 1.

Despite the insignificant increases in IgA1 levels of the ARC group as well as in IgA2 levels of the LAD group compared to HMX controls, significant elevations of total

IgA were present in every group of HIV-infected individuals. Overall, 58% of the patients examined had total IgA levels >2.9 mg/ml, 3 SD above the mean of the HTX control population. These elevations did not correlate with CD4 count or AZT therapy.

The finding that elevated IgA levels occur early in HIV infection and remain persistently high throughout different stages of disease is consistent with results obtained from analysis of serum samples available from 8 patients who had undergone progression from at least one CDC stage to another during the course of this study. Only 1 patient, progressing from ASY to ARC, with no change in CD4 count, was observed to undergo an increase in IgA, which was due to a dramatic elevation in IgA1. In contrast, the IgA level (5.8 mg/ml) of another ASY patient decreased in both subclasses, bringing the total IgA concentration to 3.2 mg/ml, after progressing directly to AIDS. The remaining 6 patients, 3 of whom had elevated levels of IgA, were found to maintain remarkably reproducible IgA concentrations over time despite having progressed as many as 2 stages, exhibiting drops in CD4 count, and, in one case, being withdrawn from AZT.

Levels of polymeric IgA

Using an ELISA assay based on the presence of J chain to distinguish polymeric from monomeric forms of IgA, sera from patients representing different stages of HIV disease were examined for possible aberrancies in the concentrations of polymeric IgA. None were found. Absolute concentrations of polymeric IgA1 and IgA2 in the sera of patients from all stages were within normal ranges (data not shown). However, since these levels remained unaffected despite increases in total IgA1 and IgA2, the percentages of serum polymeric IgA were reduced in HIV-infected individuals (Fig. 2). It is reasonable, therefore, to conclude that the elevations observed within these subclasses are represented by a corresponding increase in monomeric forms of IgA.

Presence of HIV-reactive IgA antibodies

All 22 IgA samples isolated from the serum of HIV-infected individuals were found by Western blotting to react with HIV proteins (Figs. 3-5). A summary of the IgA

FIG. 1. IgA1 and IgA2 concentrations present in the serum of HIV-infected individuals as well as heterosexual (HTX) and homosexual (HMX) controls. Each group, as described in Materials and Methods was represented by 20 patients, with the exception of HMX, which consisted of 10 seronegative volunteers. Results are expressed as geometric means ± SD.

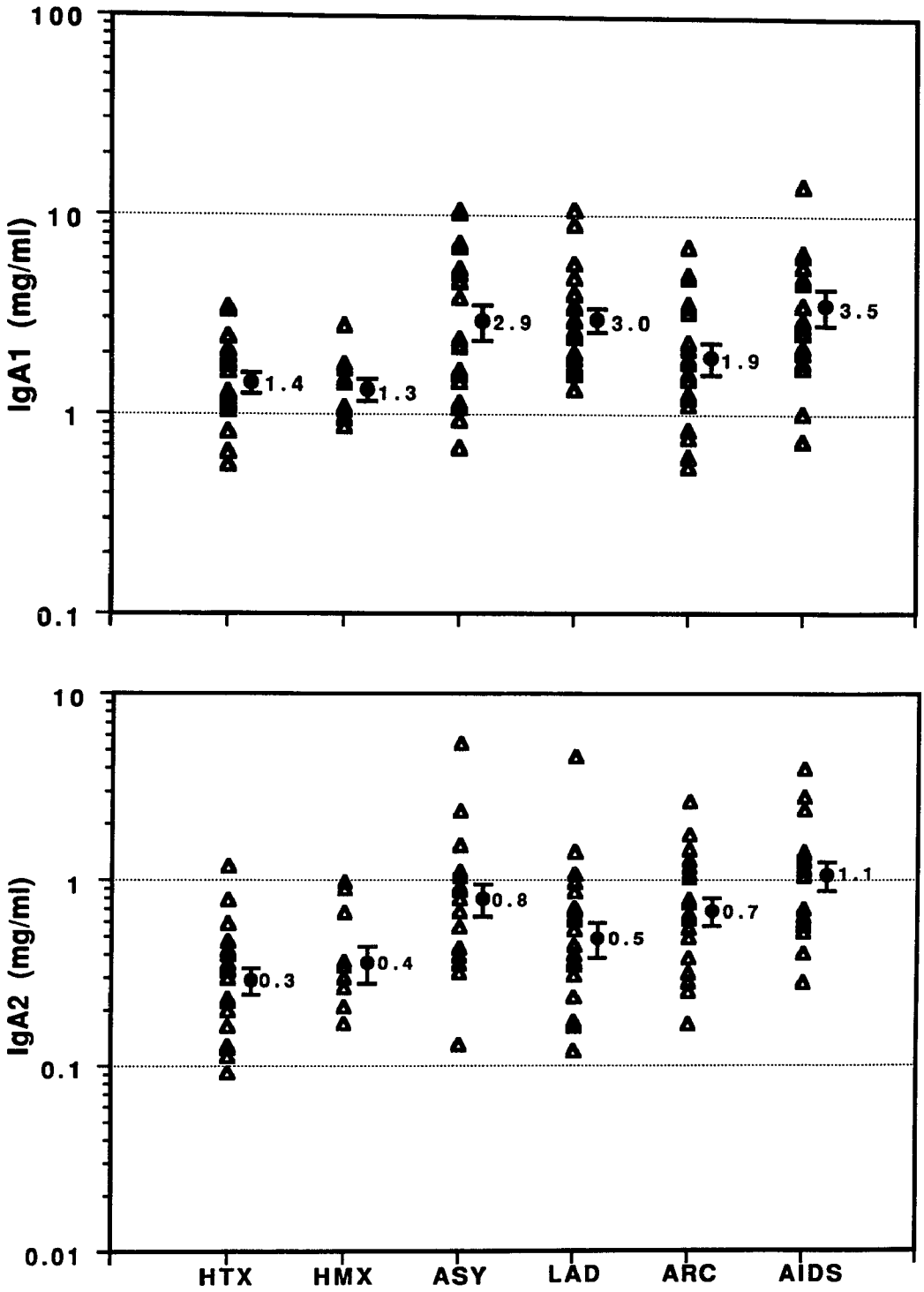

100
10
1
0.1
IgA1 (mg/ml)
1.4
1.3
2.9
3.0
1.9
3.5
10
1
0.1
0.01
IgA2 (mg/ml)
0.3
0.4
0.8
0.5
0.7
1.1
HTX
HMX
ASY
LAD
ARC
AIDS

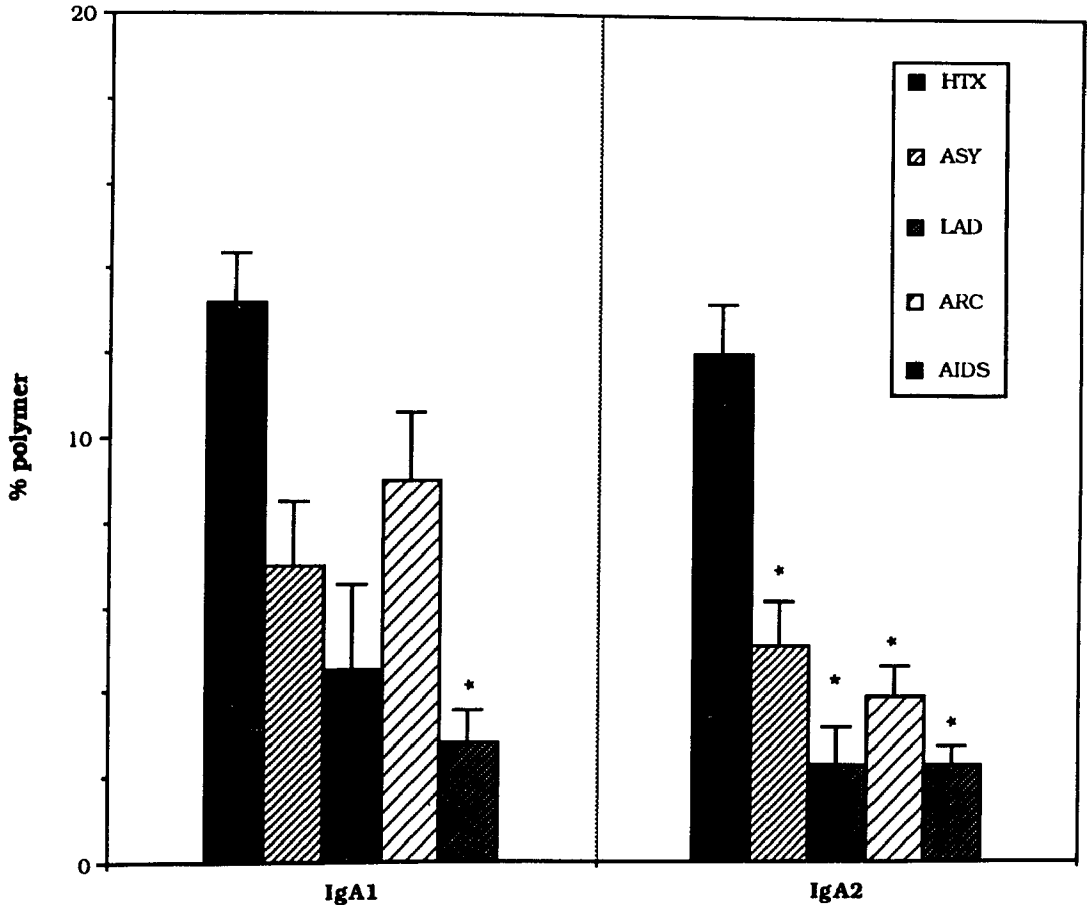
TABLE 1. P Values obtained on statistical comparison of mean IgA, IgA1, and IgA2 levels between groups[a]

Groups	IgA	IgA1	IgA2
HTX and ASY	0.0002	0.0014	0.0001
HTX and LAD	0.0005	0.0011	0.0309
HTX and ARC	0.0293	NS[b]	0.0005
HTX and AIDS	0.0001	0.0001	0.0001
HMX and ASY	0.0014	0.0037	0.0076
HMX and LAD	0.003	0.0029	NS
HMX and ARC	0.0593	NS	0.0301
HMX and AIDS	0.0001	0.0004	0.0003
ASY and ARC	NS	0.0594	NS
LAD and ARC	NS	0.0484	NS
AIDS and ARC	NS	0.0063	NS
LAD and AIDS	NS	NS	0.0013

[a]All other comparisons were nonsignificant.

[b]NS, nonsignificant.

FIG. 2. Percentage of polymeric IgA1 and IgA2 in the serum of HIV-infected individuals and control groups. Each group was represented by the following number of individuals: 20 for HTX and AIDS, 8 for ASY, 4 for LAD, and 11 for ARC. Significant differences as compared to the HTX control group are denoted by the asterix.


20
10
0
% polymer
HTX
ASY
LAD
ARC
AIDS
*
IgA1
IgA2

response to viral antigens is given in Table 2. Two dominating features of anti-HIV IgA reactivity were prevalent within individuals of all stages. First, IgA antibodies appeared to be directed primarily against the envelope glycoproteins gp160, gp120, and gp41. Every individual had IgA antibodies that reacted with gp41, while 17 in conjunction also recognized gp160/120. All of the IgA samples that did not react with gp160/120 were obtained from AIDS patients, suggesting that a declining titer of IgA antibodies against these antigens may be associated with disease progression. Second, a notable lack of reactivity to *gag* proteins p55, p24 and p18, and the *pol*-derived p32 endonuclease/integrase was observed. Of the samples examined only 4 were positive for antibodies to p31, 4 for antibodies to p18, and 10 for antibodies to p24. Even in these instances, reactivity was poor or only barely discernible. A decrease in p24-specific IgA antibodies may also accompany disease progression, since IgA of fewer ARC and AIDS patients showed reactivity against this antigen. These characteristics were confirmed (not shown) by parallel testing of samples on HTLV-III_B preblotted strips purchased from other manufacturers (Organon-Teknika, Durham, NC and BioRad, Hercules, CA).

The absence of IgA specificity for HIV *gag* and *pol* proteins was quite striking when compared to the reactivity of IgG isolated from the same patients (Fig. 4). For example, IgA antiviral activity of patient 8 was directed almost exclusively toward gp41 (Fig. 3, lane j). IgG isolated from this same patient, however, reacted against all HIV proteins, particularly p24 (Fig. 4, lane c). Similarly, IgG of patient 4 bound strongly to all viral proteins (Fig. 4, lane b), but IgA binding was restricted primarily to gp120 (Fig. 3, lane f). Recognition of reverse transcriptase (p66, p51) by IgA was observed in most patients (13/22 for p66, 8/22 for p51), although these bands also appeared to be considerably less intense than those seen with IgG antibodies.

IgA1 subclass restriction in recognition of HIV

When IgA1 and IgA2 subclass responses to HIV were investigated, IgA1 banding patterns were found to coincide with those observed for IgA. However, negative results

FIG. 3. Western blot analysis of IgA (lanes b-d) and IgA1 (lanes e-n) anti-HIV reactivity in IgA samples purified with jacalin from the serum of HIV seropositive individuals. A positive control serum from an AIDS patient was reacted with blotted HIV antigens and developed with biotinylated anti-IgG is shown in lane a. This AIDS patient responded weakly against p24. Patients 1, 4, and 5 were clinically diagnosed as ASY, patient 6 as ARC, and patients 2, 3, and 7-12 as AIDS. Due to insufficient resolution of gp160 and gp120, reactivity at this position is denoted as gp160/120 in the text. No IgA or IgG response to HIV antigens was observed with serum or purified antibodies obtained from seronegative individuals (not shown).

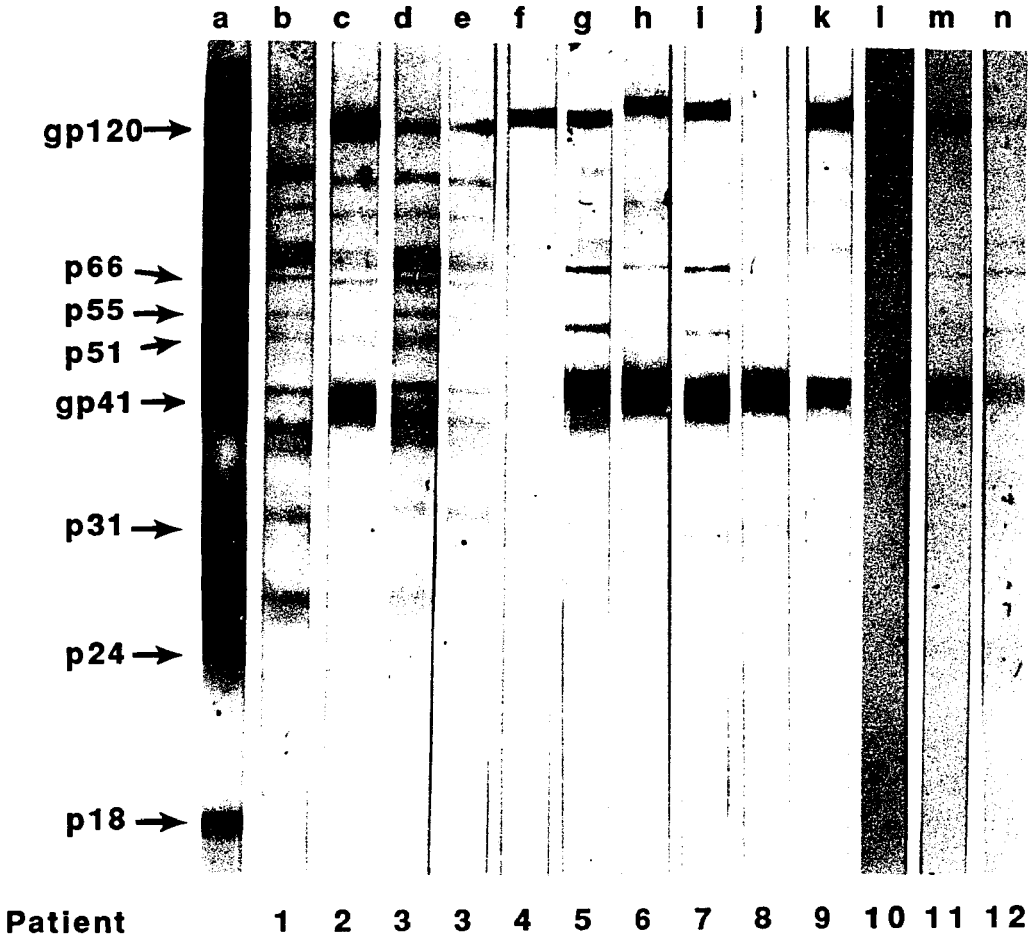
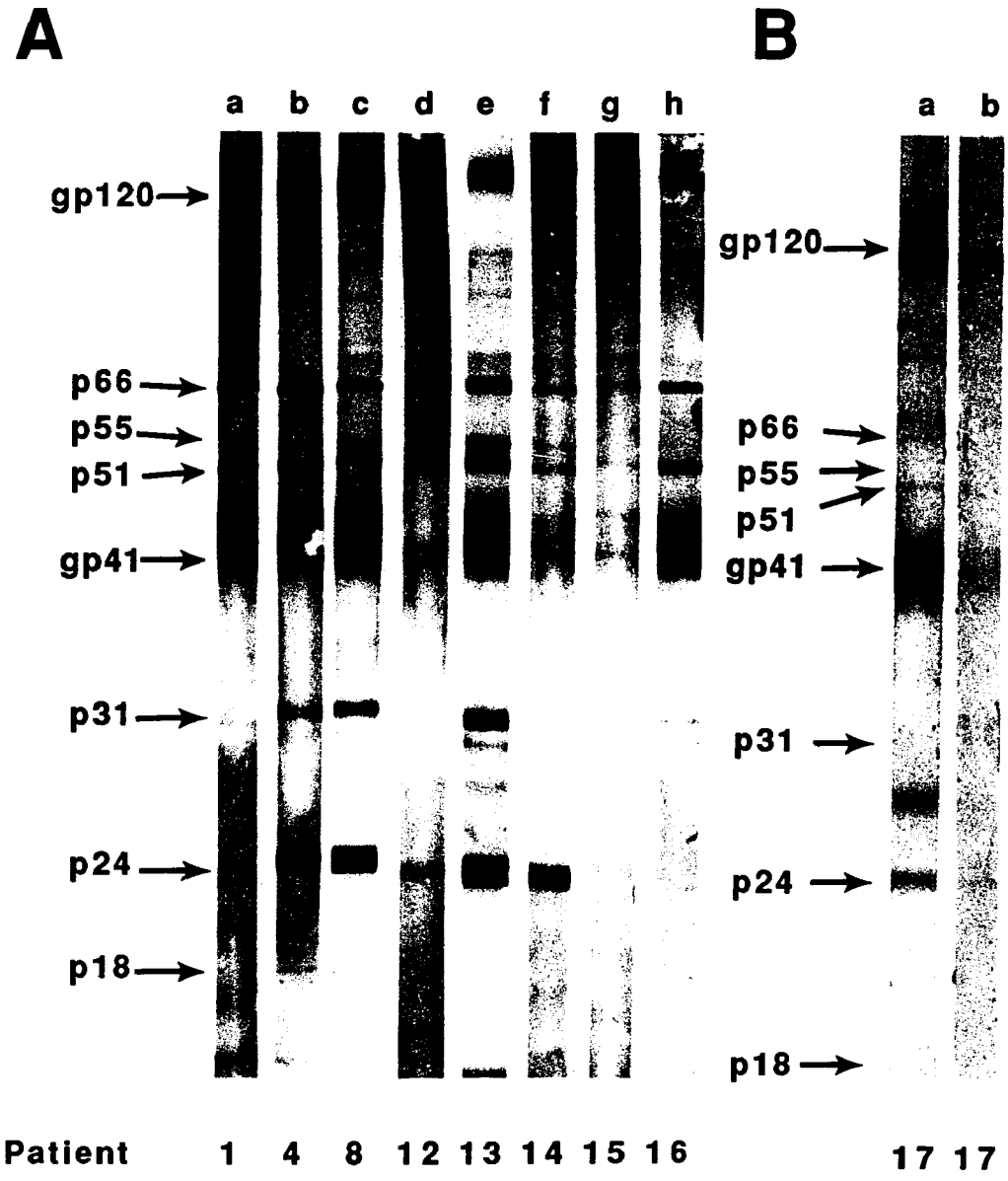

a b c d e f g h i j k l m n
gp120
p66
p55
p51
gp41
p31
p24
p18
Patient 1 2 3 3 4 5 6 7 8 9 10 11 12

FIG. 4. Western blot analysis of IgG anti-HIV reactivity present in (A) purified IgG samples from HIV-infected individuals diagnosed as ASY (patients 1, 4, 14, 15) and AIDS (patients 8, 12, 13, 16). (B) Comparison of HIV-specific antibodies in affinity-purified IgG (lane a) and IgA (lane b) samples isolated from the serum of an AIDS patient.


A
B
a b c d e f g h
a b
gp120
p66
p55
p51
gp41
p31
p24
p18
gp120
p66
p55
p51
gp41
p31
p24
p18
Patient 1 4 8 12 13 14 15 16 17 17

were consistently obtained when IgA samples were examined for IgA2 anti-HIV reactivity (Fig. 5). The presence of IgA2 in the IgA samples was confirmed by quantitative ELISA (data not shown). The possibility that IgA1, generally constituting 75-85% of total serum IgA, might be blocking IgA2 binding to HIV seems unlikely since the only sample which did give a positive IgA2 response contained 88% IgA1 (Fig.5, lanes j and k). In addition, purified preparations of IgA2, as well as IgA samples exhibiting above average percentages of IgA2, showed no IgA2 HIV reactivity (Fig. 5A, lanes f, q, and u-x).

In addition to serum IgA, two S-IgA preparations derived from colostrum of HIV-positive asymptomatic females were tested for HIV reactivity (Fig. 5B). Despite the larger proportion of IgA2 present in these samples (75% and 55%), reactivity within this subclass was only faintly observed against gp41 and several non-HIV proteins. The IgA1 response to gp41 was more intense and faint reactivity to gp160/120 and p24 was also observed.

Discussion

B-lymphocyte abnormalities are a classical manifestation of HIV disease.[10-12] Hypergammaglobulinemia and elevated levels of immune complexes were noted in the very early reports of the epidemic.[12-17] Autoimmune phenomena also are common.[10] It is clear that the IgA isotype is often strikingly elevated in AIDS patients,[12-17] and IgA levels have even been proposed as a surrogate marker of disease progression.[14] We[20] and others[21] have shown that IgA is an important constituent of the circulating immune complexes in AIDS; we further demonstrated that the IgA within the immune complexes was exclusively IgA1.[20] IgA1 autoantibodies, manifested as IgA1 rheumatoid factor, can also be found in patients with AIDS.[22] Thus, it is appropriate to emphasize that many of the common B-cell aberrancies of this disease extend to the IgA isotype and appear to be uniquely restricted to the IgA1 subclass.

The present findings indicate that increased production of serum IgA occurs very early in HIV infection, even prior to onset of clinical symptoms. Further, our data do not support the notion of a mucosal origin for elevated IgA in this disease since elevations in

FIG. 5. Western blot comparison of IgA1 and IgA2 anti-HIV reactivity in IgA samples purified by affinity chromatography from (A) the serum of HIV-infected individuals and (B) from colostrum of HIV-seropositive ASY women. An AIDS-positive control serum, developed with biotinylated anti-IgG, is shown (A: lane a). Purified IgA2 from patients 3 and 4 (see Fig. 1) was also analyzed for IgA2 reactivity (A: lanes v and x). The specific IgA subclass for which HIV reactivity was analyzed and the percent of IgA2 present in each IgA sample, as determined by ELISA, are shown. Patients 1, 4, 14, and 15 were diagnosed as ASY, patient 18 as LAD, and patients 3, 13, 16, and 19-22 as AIDS.

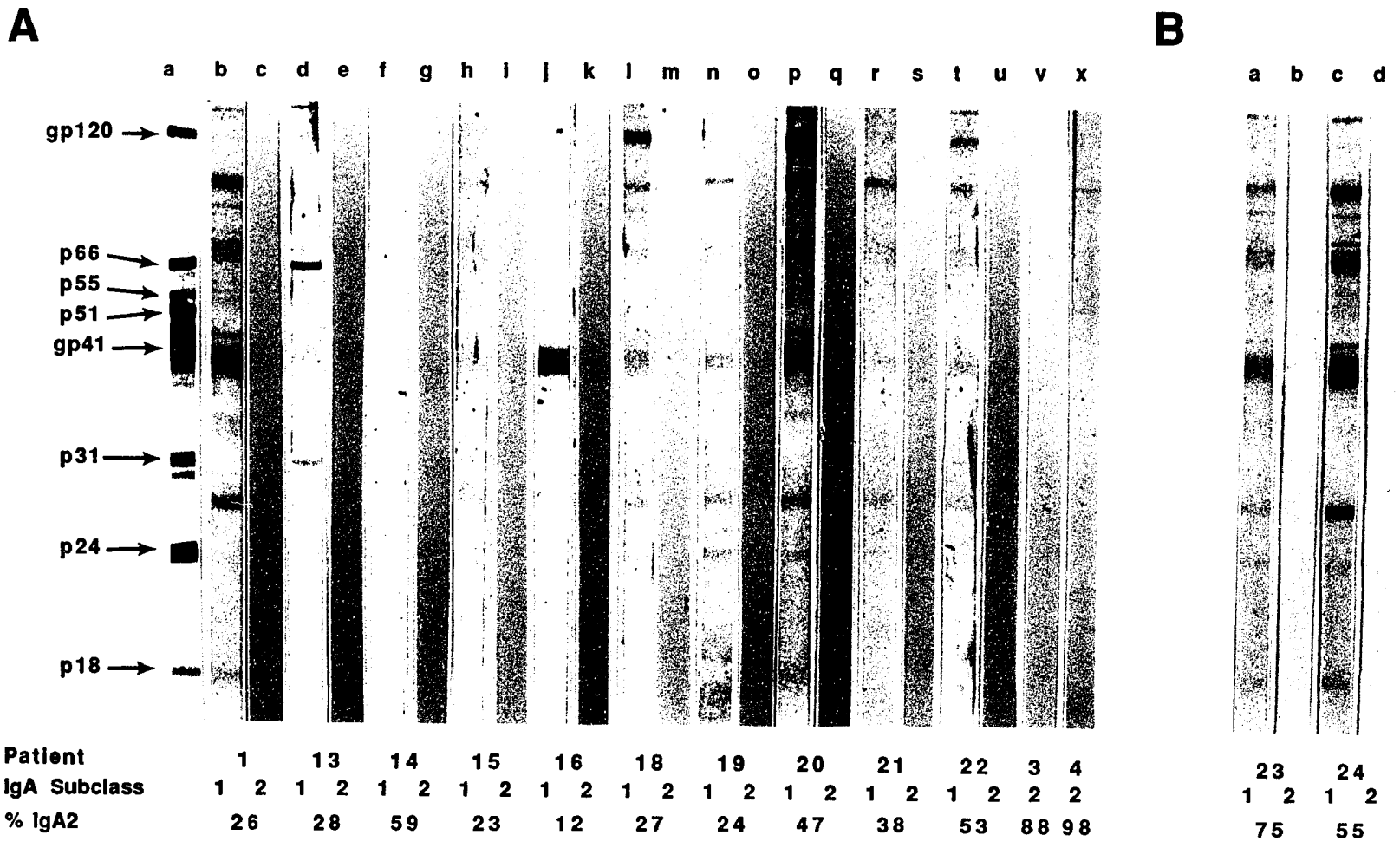

A
a b c d e f g h i j k l m n o p q r s t u v x
gp120
p66
p55
p51
gp41
p31
p24
p18
Patient 1 13 14 15 16 18 19 20 21 22 3 4
IgA Subclass 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 2 2
% IgA2 26 28 59 23 12 27 24 47 38 53 88 98
B
a b c d
23 24
1 2 1 2
75 55

TABLE 2. IgA Reactivity to HIV proteins

		Number of IgA samples reacting with						
Group	n[a]	gp160/120	p66	p51	gp41	p32	p24	p18
ASY	5	5	1	1	5	1	3	1
LAD	1	1	1	0	1	0	1	0
ARC	2	2	2	2	2	1	0	1
AIDS	14	9	9	5	14	2	6	2

[a]number of patients from whom purified IgA was tested

serum IgA are confined to the monomer fraction, the source of which is primarily the bone marrow.[5] Elevations in both IgA1 and IgA2 were exhibited as early in disease as the ASY stage and remained elevated throughout following stages. In agreement with recent work in which serum IgA subclass distributions in AIDS patients were analyzed by radial immunodiffusion,[23] we observed proportional increases in both IgA1 and IgA2. In addition, we found an alteration in the distribution of molecular forms present within both serum IgA subclasses. Despite exhibiting normal concentrations of polymeric IgA1 and IgA2, the percentage of polymer constituting each subclass was lower in the serum of HIV-infected individuals. Therefore, increases in IgA appear to be due to increases in production of monomer, which cannot be directly assayed.

Studies indicate that abnormal regulation of IgA synthesis in secretory tissues also occurs with HIV infection. In AIDS patients, selective decreases in salivary IgA have been reported.[24,25] These results suggest that HIV infection may result in differential effects on IgA production in the systemic and mucosal compartments. The role of both serum and mucosally derived S-IgA in HIV infection remains unknown. HIV-specific S-IgA detected in saliva, semen, milk, and cervicovaginal washes may function to eliminate virus at

mucosal sites.[6] However, the prevalence of impaired mucosal responses and the presence of HIV in these secretions[26-29] suggest that, if present, S-IgA neutralizing function is inadequate in providing protection from disease.

Detection of HIV-specific IgA antibodies in serum by Western blotting has sometimes been difficult, presumably because of interference by higher concentrations of IgG antibodies.[30] Using purified IgA, HIV specificity has clearly been demonstrated. As shown here, serum IgA1, but not IgA2, also reacts with HIV-1 proteins. The characteristics of this response, however, appear to be quite different from those observed with IgG. While IgG antibodies broadly reacted to all HIV antigens, IgA1 antibodies predominantly recognized envelope glycoproteins. In many cases, striking IgG reverse transcriptase- and p24-specific antibody responses were accompanied by a total absence of reactivity to these antigens within the IgA isotype. In addition, fewer responses to gp160, gp120, and p24 were observed by IgA purified from ARC and AIDS patients. A loss of IgA antibodies specific for these antigens may accompany disease progression.

Although the consequences of HIV-directed IgA1 antibodies are unclear, the predominance of these antibodies targeted for gp160/120 and gp41 implies a potential for neutralization of virus. However, IgA, unlike IgG, does not activate complement.[31] Instead, complement-mediated effector mechanisms can be blocked by antigen-specific IgA.[7-9] This anti-inflammatory property of IgA has led to the suggestion that this isotype may function beneficially as a regulatory mediator. On the other hand, it is conceivable that this property may actually hinder IgG-directed lytic responses to HIV, particularly in individuals with elevated IgA.

The presence of IgA1 responses primarily specific for gp41 coincident with the inability to demonstrate HIV IgA2 reactivity in the colostral IgA samples examined suggests that the targeting of *env* proteins and restriction of these responses to the IgA1 subclass may also be present in the secretions of HIV-infected individuals. This restriction of serum anti-HIV IgA antibodies to the IgA1 subclass is not entirely unexpected. Serum

IgA antibodies to viral, bacterial and food antigens are found predominantly within the IgA1 subclass.[32] Studies which have analyzed subclass distributions in saliva suggest that exposures to most protein and neutral carbohydrate antigens tend to elicit an IgA1 response.[32] IgA2 antibodies, on the other hand, are often directed at highly antigenic amphiphilic bacterial components, such as lipopolysaccharide, lipoteichoic acid, and other polysaccharide antigens.[32,33]

The inability to demonstrate IgA2 anti-HIV specificity gives rise to the question of function of the excessive amounts of serum IgA2 produced in HIV-infected individuals and the factors involved leading to the activation of IgA-committed B cells. Increases in serum Ig may be generated specifically in response to HIV, as suggested by *in vitro* studies showing that 20-40% of the immunoglobulin spontaneously produced by PBL from infected individuals is HIV-specific.[34] Though we did not quantitate HIV-specific IgA1, elevations in this subclass may be at least partly due to a specific response to viral infection. However, the presence of significantly elevated IgA2 not reactive with HIV indicates other factors must contribute to increased serum IgA levels. Several hypotheses regarding the origin of a polyclonal B-cell activation and hypergammaglobulinemia in HIV infection have been proposed. These include a direct stimulation of B cells by HIV, the reactivation of Epstein-Barr virus (EBV) in B cells latently infected with this virus, and infection of EBV-transformed B cells with HIV itself.[35] The possibility also exists that anti-HIV antibodies themselves may participate in B-cell activation. A region of the HIV envelope glycoprotein, gp120, has been found to share 34% homology with the first constant domain (CH1) of the human $\gamma 2$ heavy chain and, to a similar extent, with this domain of the $\gamma 1$ and $\gamma 4$ heavy chains.[36] Interestingly, another region of gp120 exhibits 30% homology with the CH1 domain of IgA2.[36] If anti-gp120 antibodies produced by infected individuals are also capable of recognizing membrane IgG and IgA2 expressed on mature B cells, cross-linking of this surface Ig could conceivably initiate events leading to cellular activation. Though the potential for reactivity against IgA2 has not been examined, some

evidence for the production of antibodies cross-reactive with IgG has come from the finding that the serum of more HIV-infected individuals reacts with a γ chain peptide comprising this domain than do the sera of seronegative individuals.[37] Further studies will be required to determine whether autoimmune phenomena actually exist in vivo and contribute toward elevating serum immunoglobulin levels in individuals infected with HIV.

Acknowledgments

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CONTRASTING IgA AND IgG NEUTRALIZATION CAPACITIES AND RESPONSES TO HIV TYPE 1 GP120 V3 LOOP IN HIV-INFECTED INDIVIDUALS

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Abstract

Quantitative analysis for HIV-1-specific antibodies present in IgA and IgG preparations purified from the serum of HIV-seropositive individuals indicated that the proportion of HIV-specific antibodies present within the IgG isotype was seven times greater than the proportion of IgA HIV antibodies present within the IgA isotype. Dilution of IgA HIV-specific antibodies by nonspecific IgA was observed in patients with elevated serum IgA concentrations, whereas proportions of IgG HIV antibodies rose with increases in concentrations of serum IgG. Though proportions of IgA HIV antibodies were not observed to correlate with the CD4 counts of the individuals from whom immunoglobulins were purified, a significant association between the numbers of such cells and proportion of HIV antibodies present in the IgG isotype was found. Equivalent amounts of IgG were also more effective than IgA at inhibiting HIV-1_{IIIB} infection of a susceptible T cell line. This may be due to the presence of higher proportions of IgG antibodies directed toward non-V3 determinants since reactivity against an HIV-1_{IIIB} V3 peptide was very low and did not differ significantly between these isotypes. IgA antibodies reacting against a V3 peptide containing the HIV consensus sequence could be detected in the majority of IgA samples purified from infected individuals. Proportions of IgG consensus V3-specific antibodies within the purified IgG samples were, however, much higher. The presence of accompanying increases in serum IgG concentration and proportions of IgG HIV antibodies, higher proportions of both HIV- and consensus V3-specific antibodies within this isotype, and more effective neutralization by IgG suggests that an HIV-driven response is dominated by B cells committed to production of this immunoglobulin isotype. The observed low proportions of HIV antigen-specific IgA antibodies with dilution in many individuals by elevations in non-HIV-specific IgA suggests that IgA B cells may be more susceptible to factors which mediate the polyclonal activation believed to be responsible for many of the B cell disorders characteristic of HIV infection.

Introduction

Unlike antibodies produced in other viral infections, neutralizing anti-HIV antibodies developed by the host do not effectively eradicate virus. The reasons for this are unknown, but may involve a failure of B cells to appropriately respond to emerging viral variants.[1] It is evident that prior to development of clinical symptoms or significant loss of CD4+ T cells, HIV-infected individuals have profound B cell abnormalities. This has been demonstrated by depressed proliferative responses to both T-independent and T-dependent mitogens *in vitro* and reduced antibody titers against protein and polysaccharide antigens *in vivo* following immunization (for review, see ref. 2). In the circulation, hypergammaglobulinemia coupled with elevated levels of autoantibodies and antibodies against other pathogens parallels an increase in the number of activated B cells and lymphocytes spontaneously producing immunoglobulin.[2] Taken together, these findings are indicative of an intense polyclonal activation of B cells. Nevertheless, the induction of an HIV-specific immune response is also suggested by the finding that as much as 40% of immunoglobulin (Ig) spontaneously secreted by lymphocytes is directed against HIV.[2] That HIV-driven responses do accompany infection is also supported by the presence of extensive somatic mutation in anti-HIV IgG antibodies.[3,4]

Most of the studies examining antibody function in HIV infection have utilized reagents for detection of IgG or total Ig. In addition, by virtue of its predominance over other isotypes in serum, antibody-mediated cellular cytotoxicity and neutralizing activities previously described likely reflect properties of IgG. This is illustrated by the difficulties encountered in demonstrating serum IgA anti-HIV antibodies by western blotting prior to the removal of IgG.[5] Therefore, it is still not clear what functional role IgA may play or how IgA B cells respond in HIV infection. Aberrancies are suggested by the presence of IgA hypergammaglobulinemia in the serum of many seropositive patients.[6] This response is likely polyclonal in nature since both IgA subclasses are elevated, despite a restriction of HIV-specific antibodies to the IgA1 subclass.[6]

By western blotting and development with affinity-purified preparations of IgA and IgG isolated from the serum of HIV-infected patients, we have observed dramatic differences between these two isotypes with regard to their reactivity against HIV.[6] IgG reacted broadly against all HIV antigens, while IgA almost exclusively recognized envelope glycoproteins. During this qualitative analysis, we noted that higher concentrations of IgA than IgG were necessary to demonstrate binding to HIV. Although this could reflect differences in detecting reagents, it is also possible that of the total serum IgA produced by infected individuals, a lower proportion of IgA HIV antibodies is produced in comparison to the proportion of HIV-specific antibodies present within the IgG isotype. A dilution of HIV-specific IgA antibodies by increases in nonspecific IgA could potentially lead to lower proportions of circulating IgA HIV antibodies, particularly in patients with elevated IgA. If fewer HIV IgA antibodies are present, including those directed against the HIV principal neutralizing domain (PND), IgA would be expected to be less effective at neutralizing HIV infection than an equivalent amount of IgG.

To determine whether plasma of HIV-infected individuals may indeed contain lower proportions of HIV IgA antibodies within total IgA as compared to those present within IgG, we have purified both IgA and IgG from the serum of seropositive patients and quantitatively assayed each isotype for HIV-specific antibodies by ELISA. The results were then examined relative to the concentration of total circulating IgA and IgG to determine if any association between proportions of HIV antibodies and hypergammaglobulinemia existed within the isotypes. In addition, we tested equal amounts of purified IgA and IgG in a neutralization assay to elucidate whether IgA, like IgG, can protect a susceptible T cell line from HIV-1_{IIIB} infection. Ability of IgA or IgG to neutralize HIV was then analyzed in relation to proportions of HIV-1_{IIIB} V3-specific antibodies quantitated within these samples by ELISA. Finally, to determine whether IgA neutralizing antibodies are produced in response to HIV infection, IgA samples were also quantitatively assayed for proportions of V3-specific antibodies using a gp120 V3

loop peptide containing the North American consensus sequence for the PND, since the majority of our patient population is not likely to have been exposed to the IIIB isolate.

Materials and Methods

Serum and immunoglobulin purification

Serum samples from 32 HIV-seropositive individuals were obtained from the University of Alabama (UAB) Center for AIDS Research Clinical Core Facility and stored at -70°C until use. Fifteen subjects were classified by CDC staging guidelines as asymptomatic (Stage II) whereas the remaining 17 were diagnosed with AIDS (Stage IVC-1). In addition, serum was obtained from 22 healthy seronegative volunteers. IgG and IgA were purified from serum by affinity chromatography as previously described,[6] using Protein G (Pharmacia, Piscataway, NJ) and a monoclonal antibody specific for both human IgA subclasses. Isolated IgG and IgA were dialyzed against phosphate-buffered saline (PBS), sterilized by 0.22-μm (pore size) filtration, and analyzed for immunoglobulin content by ELISA. Purity ranged from 99-100% for IgG and 96-100% for IgA.

ELISA for total IgG and IgA

Total IgG in serum and purified Ig samples was measured on 96-well microtiter plates (Dynatech, Chantilly, VA) coated with affinity-purified $F(ab')_2$ fragments of goat anti-human IgG (Rockland, Gilbertsville, PA) as previously described.[7] Moni-Trol Ig reference serum standard (Baxter, Miami, FL) was assayed simultaneously with unknowns on all plates. After consecutive treatments with biotinylated goat $F(ab')_2$ specific for human IgG (Tago Inc., Burlingame, CA) and avidin-labeled peroxidase (Sigma, St. Louis, MO), plates were developed with 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and H_2O_2 enzyme substrate. Absorbance at 414 nm was recorded in an automated plate reader (Anthos Reader 2001, Bioquip, Atlanta, GA) and sample concentrations determined from calibration curves constructed by a computer program based on four-parameter logistic algorithms. IgA concentrations

were quantitated as described[6] using plates coated with monoclonal antibodies specific for either IgA1 or IgA2, the generous gift of J. Radl (IVVO-TNO, Leiden, The Netherlands). Plates were developed after subsequent incubations with biotinylated goat anti-human IgA $F(ab')_2$ (Tago), alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) and phosphatase substrate (Sigma). Concentration of IgA1 and IgA2 in unknowns was calculated as above from absorbance values at 405 nm. Total IgA was determined by combining IgA1 and IgA2 concentrations.

Cells and virus

The U937 promyelomonocytic and CEM T cell lines were maintained in RPMI (GIBCO, Gaithersburg, MD) supplemented with penicillin-streptomycin and 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT). A viral stock for neutralization assays was generated by infecting CEM cells with $HIV\text{-}1_{IIIB}$ (kindly provided by R. W. Buckheit Jr., Southern Research Institute, Frederick, MD). Five days postinfection, cells were removed from cultures by low-speed centrifugation and media cleared of debris by 0.45-μm (pore size) filtration, aliquotted, and frozen at -70°C. Cells were then resuspended in fresh media and allowed to incubate another 24 h. Potential stocks were produced in this manner for 3 consecutive days. The infectious activity of each stock was then determined by endpoint titration[8] with slight modifications. Briefly, quadruplicate cultures containing 1×10^5 (100μl) U937 or CEM T cells in 24 well plates (CoStar, Cambridge, MA) were inoculated with 10-fold serial dilutions of virus (100μl) in media at 37°C. After 1 h, media was added to bring the final volume to 1 ml. Cells were then maintained in culture for 21 days. Throughout this period, a reverse transcriptase (RT) assay was used to monitor viral production. The tissue culture infectious dose (TCID) was determined on day 21 and defined as the reciprocal of the last dilution capable of inducing infection in a minimum of 50% of the cultures receiving that inoculum. The stock chosen for use was determined to have 10^5 $TCID_{50}$/ml on both the U937 and CEM cell lines.

HIV virions were purified for ELISA according to established protocols.[9] Briefly, virus produced by HIV-1_{IIIB}-infected U937 cells was purified at 3 day intervals from sterile-filtered culture media by ultracentrifugation at 28,000 rpm for 2 h at 4°C. Pelleted virions were resuspended in 20 mMTris (pH7.5), 100 mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA) and further purified by column chromatography on Sepharose 4B (Sigma). Eluted virus was again pelleted, lysed in Dulbecco's PBS (DPBS; Mediatech, Norcross, GA) containing 0.1% Triton X-100, and frozen at -70°C. Concentrated viral lysates generated from 2L of culture medium were combined to give a final volume of 1 ml, 37.5µl of which was determined to be optimal in 10 ml PBS for coating 1 ELISA plate. A stock of concentrated U937 cell proteins was also prepared for the coating of adsorption plates. This entailed pelleting 1L of culture medium containing uninfected U937 cells, resuspending cells in 5 ml DPBS and freeze-fracturing twice on a dry ice-ethanol bath as described.[9] Adsorption plates were coated with a solution containing 350µl of this preparation that had been diluted into 10 ml DPBS.

Synthetic peptides

A 35 amino acid peptide containing the gp120 V3 North American consensus sequence of HIV-1 (CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC) with an additional cysteine residue at the carboxy terminal to facilitate later conjugation was generously provided by G. M. Anantharamaiah (Department of Medicine, UAB). Following synthesis of the peptide on an Advanced Chemtech (Louisville, KY) automatic peptide synthesizer, an intramolecular disulfide bond between the two cysteine residues was formed by air oxidation in the presence of potassium ferricyanide. Conformation of the peptide sequence was accomplished through amino acid sequencing analysis and ion spray mass spectroscopy. Analysis of free sulfhydryl groups confirmed that greater than 90% of the peptides had a loop structure. The peptide was purified by reversed-phase high-performance liquid chromatography (HPLC) on C_{18} columns and conjugated to keyhole limpet hemocyanin (KLH) using N-succinimidyl-3-(2-pyridyldithio) propionate

(Pierce, Rockford, IL), which was found to greatly enhance binding to ELISA plates. The KLH-conjugated peptide was then further purified by HPLC.

The following reagent was obtained through the AIDS Research and Reference Reagent Program (NIAID, NIH): HIV-1_{IIIB} peptide 295-321 contributed by S. Pincus.[10] This HPLC-purified peptide was synthesized according to standard Fmoc chemistry on an Applied Biosystems (Foster City, CA) peptide synthesizer and represents the HIV-1_{IIIB} gp120 V3 neutralization loop (TRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAH). All peptides were stored at -70°C prior to use in immunoassays.

ELISA for HIV- and V3-specific antibodies

Maxisorp F-96 Nunc-immunoplates (USA/Scientific Plastics, Ocala, FL) were coated with HIV lysate diluted in PBS as detailed above. Polyvinyl chloride microtiter plates (Dynatech) were coated with either 0.5μg/ml of the consensus V3 peptide conjugated to KLH, KLH (1μg/ml) (for consensus V3 adsorption plates), or U937 cell lysate (for HIV adsorption plates) in PBS. Immulon II 96-well plates (Dynatech) were coated with the HIV-1_{IIIB} gp120 V3 peptide at 5μg/ml. Following an overnight incubation at 4°C, plates were washed with PBS containing 0.05% Tween (wash buffer) and blocked for 1 h at 25°C with PBS containing 5% horse serum (Sigma) and 0.05% Tween (reagent buffer). Adsorption plates were first reacted with 110μl/well of each purified IgG or IgA sample, ranging from 6.25 - 100μg/ml in reagent buffer for 2 h at 25°C. After this time, 200μl of each sample was transferred to either HIV- or V3-coated microtiter plates and twofold serially diluted. For the generation of a standard curve, optimal starting concentrations of the reference standard, which consisted of a pool of IgG purified from 5 HIV-infected individuals, were found to be 200μg/ml for assays with the HIV-1_{IIIB} V3 peptide and 50μg/ml for those with the consensus V3 peptide and HIV lysates. These starting concentrations were assigned values of 4 and 1 ELISA units/ml, respectively. Samples were allowed to react overnight at 4°C, after which plates were washed, treated with biotin-labeled goat $F(ab')_2$ anti-human Ig (Southern Biotechnology

Associates, Birmingham, AL) for 2 h at 25°C, again washed and avidin-labeled peroxidase added. After 40 min at 25°C, plates were washed, developed with ABTS-H_2O_2 substrate, and absorbance at 414nm recorded. Anti-HIV or anti-V3 ELISA units present in samples were determined by interpolation from a standard curve as outlined above. Proportions of HIV ELISA units present in IgA and IgG samples were further verified with peroxidase-conjugated rabbit anti-human κ and λ chains (Dako Corp., Carpinteria, CA) as a different detecting reagent.

Cell protection assay

In 24-well plates, triplicate samples of 100μg of IgG or IgA1 were mixed with 100 $TCID_{50}$ of HIV-1_{IIIB} and rotated for 1 h at 4°C. CEM cells (1 x 10^5) were then added and incubation continued for another 60 min at 37°C. Medium was then added to bring the final volume to 1 ml. After 4 days of incubation at 37°C in 5% CO_2, cultures were split 1:2. On day 7, cells were removed by centrifugation and culture medium frozen at -20°C for RT assay. After assay, background RT values, determined from uninfected cell controls, were subtracted from all other values. Inhibition of viral infection in cultures which received IgA1 or IgG were then calculated relative to cultures which were incubated with virus alone. IgA1 and IgG samples contained no infectious activity as determined by the absence of reverse transcriptase activity in medium obtained on day 7 from cell cultures incubated with the purified Ig alone.

Reverse transcriptase assay

Cell-free culture medium stored at -20°C was thawed and 15μl mixed with 10μl of reaction cocktail, yielding a final volume containing 40 mM Tris, 32 mM $MgCl_2$, 40 mM dithiothreitol (DTT), 200mM ethylene glycol-bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2% Triton X-100, 2.5 μCi [^{3}H]-dTTP (NEN Research Products, Atlanta, GA), and poly(rA)·$p(dT)_{12-18}$ (80 μg/ml; Pharmacia) at pH 7.6. Samples were allowed to react for 2 h at 37°C, after which 22μl was removed and placed onto a designated 1cm^2 piece of DE81 ion exchange chromatography paper (Whatman

LabSales, Hillsboro, OR). Nonspecific material was removed by washing 6 times in 5% w/v Na_2HPO_4 for 5 min, twice in distilled H_20 for 1 min, and twice for 1 min in 70% ethanol. Each paper square was then immersed in Econo-Safe counting cocktail (Research Products International, Mount Prospect, IL) and associated radioactivity determined in a scintillation counter.

Statistics

The Statview data analysis and presentation computer software package (Abacus Concepts, Inc., Berkeley, CA) was used to perform all statistical comparisons and to calculate geometric means and standard deviations. Differences between groups were determined using the nonparametric Mann-Whitney U-test. p values less than 0.05 were considered statistically significant. Linear relationships between variables, such as CD4 count and proportions of HIV-specific antibodies, were determined through correlation analysis, with conversion of correlation coefficients to p values using Fisher's r-to-z transformation. Reactivity in ELISA assays or inhibition of viral infection by samples was considered statistically significant when individual values were 3 standard deviations (SD) above the geometric mean of the relevant control group.

Results

Reactivity of IgA and IgG against HIV

To determine whether IgA and IgG produced by HIV-infected individuals contains different proportions of antibodies directed against HIV, IgA and IgG were affinity-purified from the sera of seropositive individuals, adjusted to known concentrations, and analyzed by ELISA for reactivity against whole viral lysates. As shown in Fig. 1, significantly more IgG purified from both asymptomatic (ASY) and AIDS patients bound to HIV than did IgA from the same individuals. When examined on an individual basis, infected subjects, whether symptomatic or not, had an average of 7.25-fold more ELISA units of anti-HIV/mg IgG than anti-HIV/mg IgA (data not shown). In only one subject was IgA observed to react equivalently to IgG. Reactivity of IgA and

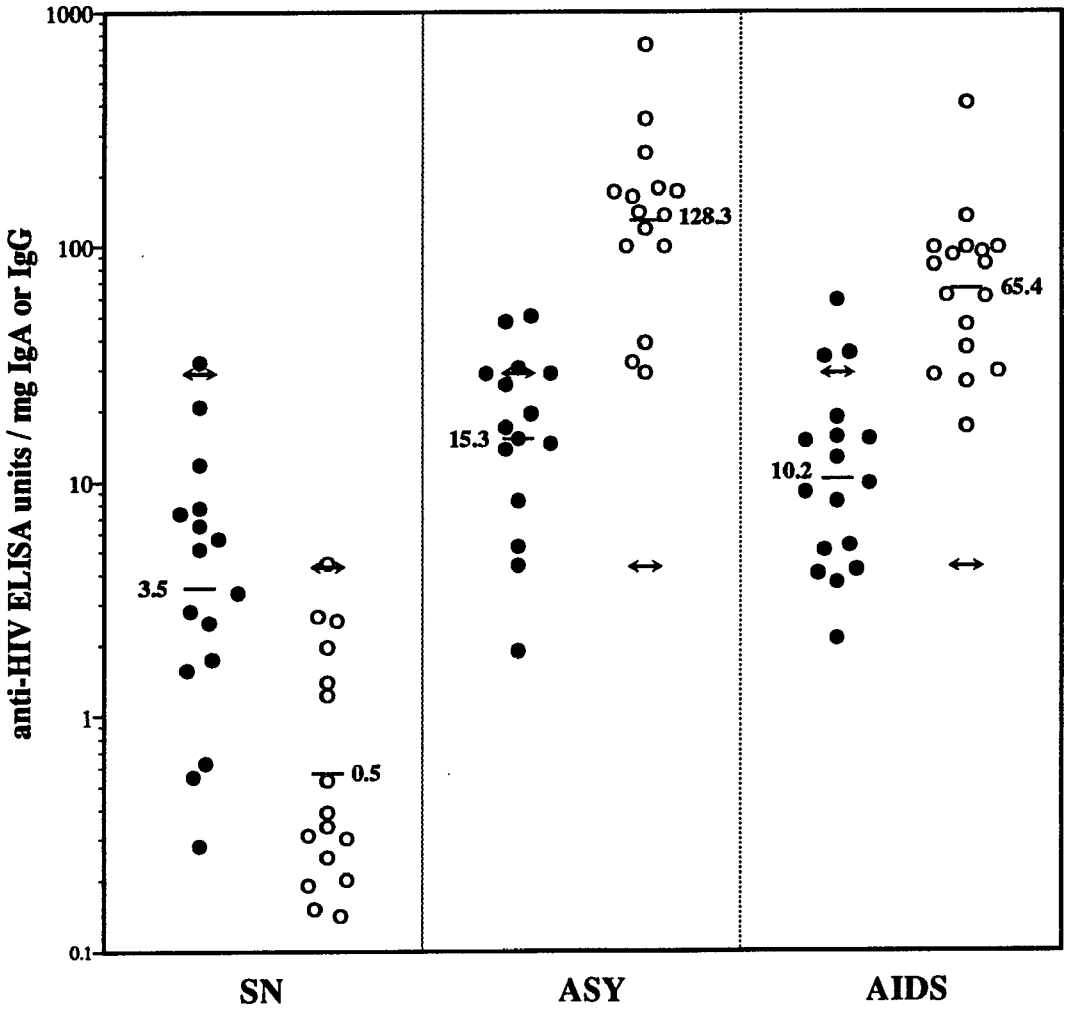

FIG. 1. Proportions of HIV-specific antibodies present within IgA (●) and IgG (o) samples purified from the sera of 16 SN individuals, 15 ASY, and 17 AIDS patients. Mean anti-HIV ELISA units per milligram of each isotype for each group are shown (—). Arrows (↔) indicate the level at which HIV ELISA units per milligram are significantly different from controls (3 SD over the mean of the corresponding SN group). With the exception of SN IgA and SN IgG, for which $p = 0.0005$, all statistical comparisons within and between groups of mean IgA with mean IgG levels gave a p value of 0.0001. Significant differences were also observed between the following: SN IgA and ASY IgA, $p = 0.0023$; SN IgA and AIDS IgA, $p = 0.0158$; SN IgG and ASY IgG, SN IgG and AIDS IgG, both $p = 0.0001$; ASY IgG and AIDS IgG, $p = 0.0049$.

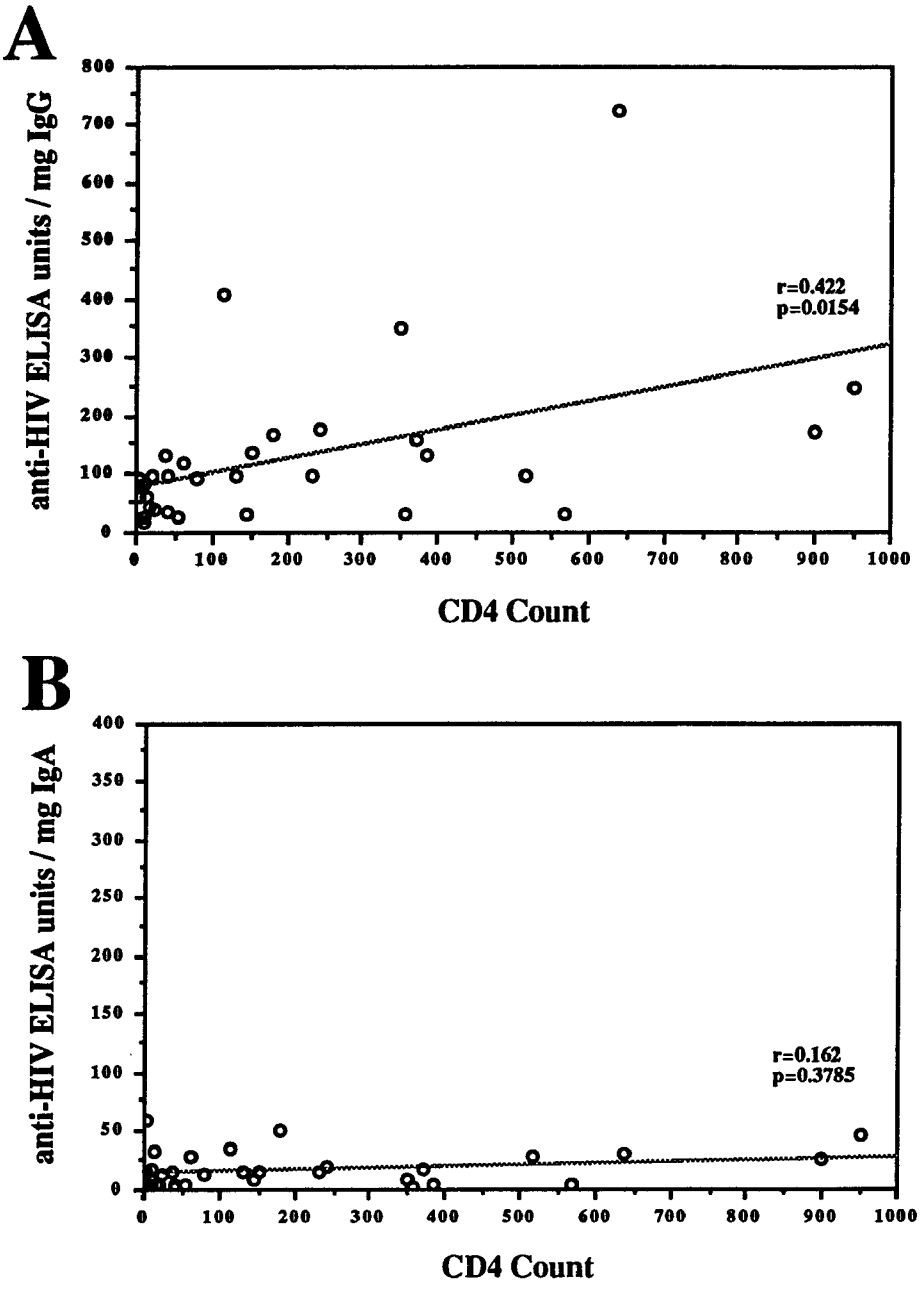

FIG. 2. Relationship of CD4 count and proportions of HIV antibodies. The proportions of anti-HIV ELISA units per milligram of (A) IgG and (B) IgA are shown in relation to the CD4 count (cells/mm^3) of the infected individuals from whose sera the samples were purified. The p values and correlation coefficients (r) were calculated as described in Materials and Methods.

IgG from seronegative (SN) controls completely contrasted with that observed in infected subjects. Seronegative IgG binding to lysates was extremely low, averaging only 0.57 ELISA units/mg IgG for the entire group. The average binding by SN IgA was significantly higher (3.5 ELISA units/mg IgA) and greater binding to lysates by IgA was detected in every SN individual. On average, SN individuals had 6.1 times higher proportions of IgA than IgG reacting with HIV lysates.

Of the 32 IgG samples analyzed, 100% reacted significantly with virus (>4.3 anti-HIV ELISA units/mg IgG; 3 SD above the mean SN IgG control value). In contrast, binding greater than 3 SD above the mean of SN IgA (29.0 anti-HIV ELISA units/mg IgA) was detected in only 20% of the ASY and 18% of the AIDS IgA samples. Nevertheless, both of these groups had IgA containing significantly higher proportions of HIV-specific ELISA units when compared to the SN group (see Fig. 1 caption). Less reactivity against HIV was noted in both isotypes purified from AIDS patients when compared to the ASY group. This led us to reexamine results in relation to the CD4 count of all study subjects. Declining CD4 count was found to be significantly associated with decreases in IgG-mediated HIV reactivity (Fig. 2A), but not with IgA mediated binding (Fig. 2B). Instead, the proportion of anti-HIV ELISA units in IgA samples was observed to correlate negatively with the serum IgA concentration of the patient ($p=0.0384$). Quantitation of the total IgA and IgG present in serum (Fig. 3) from which purified samples were obtained indicated that a higher percentage of study subjects with AIDS demonstrated IgA hypergammaglobulinemia (41%) than those subjects remaining symptomless (27%). The mean concentration of serum IgA was also higher for the AIDS group and, in contrast to the ASY group, significantly elevated when compared to SN IgA. When subjects were recategorized on the basis of normal or elevated serum levels of IgA or IgG, patients with IgA hypergammaglobulinemia were found to contain significantly fewer anti-HIV ELISA units per milligram of this isotype compared to those with normal serum IgA concentrations (Fig. 4). Therefore, poor reactivity against HIV

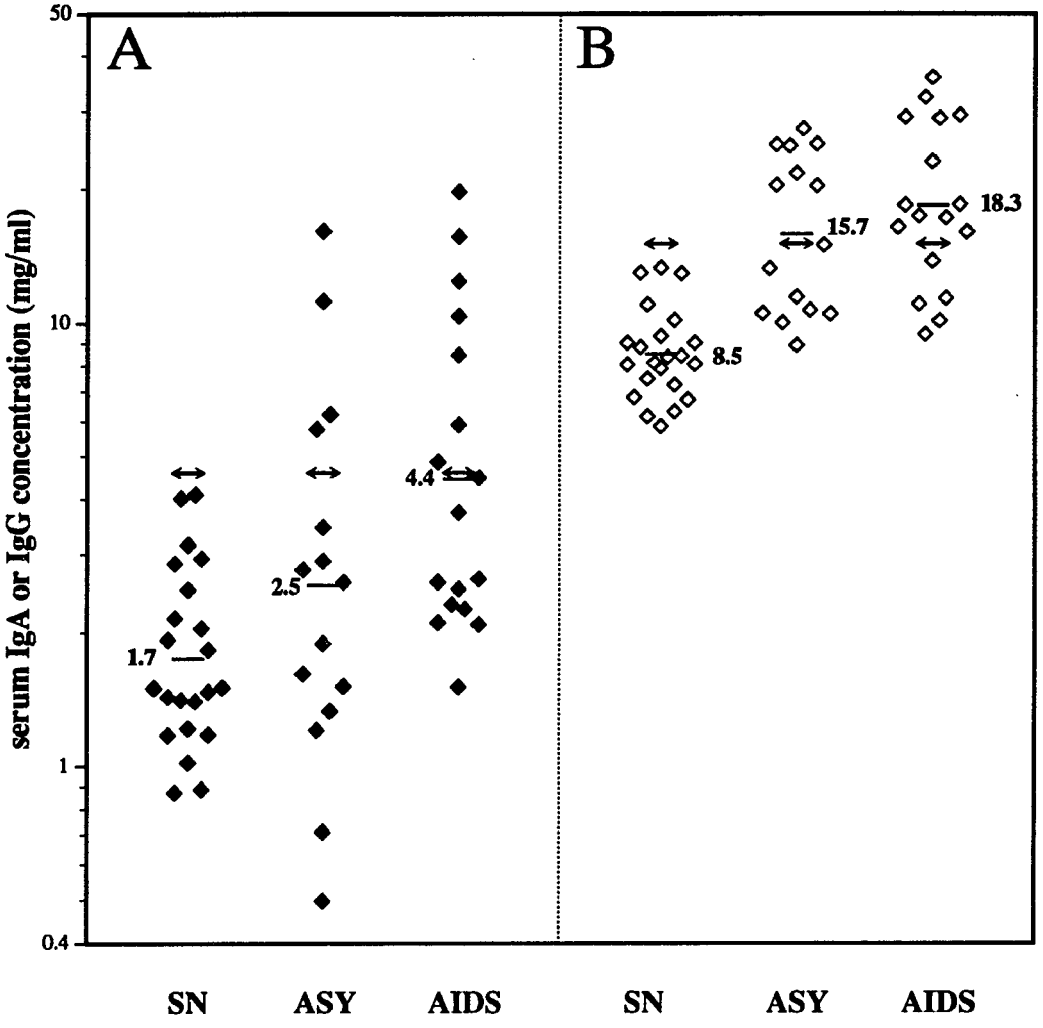

FIG. 3. IgA and IgG concentrations in the sera of HIV-infected individuals. (A) IgA (◆) and (B) IgG (◇) concentrations present in the sera of the 15 ASY and 17 AIDS study subjects and 22 SN individuals were determined by quantitative ELISA. Mean concentrations (−) for each group and levels significantly above controls (↔) are shown. Significant differences were observed between the following groups: SN IgA vs. AIDS IgA, SN IgG vs. ASY IgG, and SN IgG vs. AIDS IgG; all p = 0.0001.

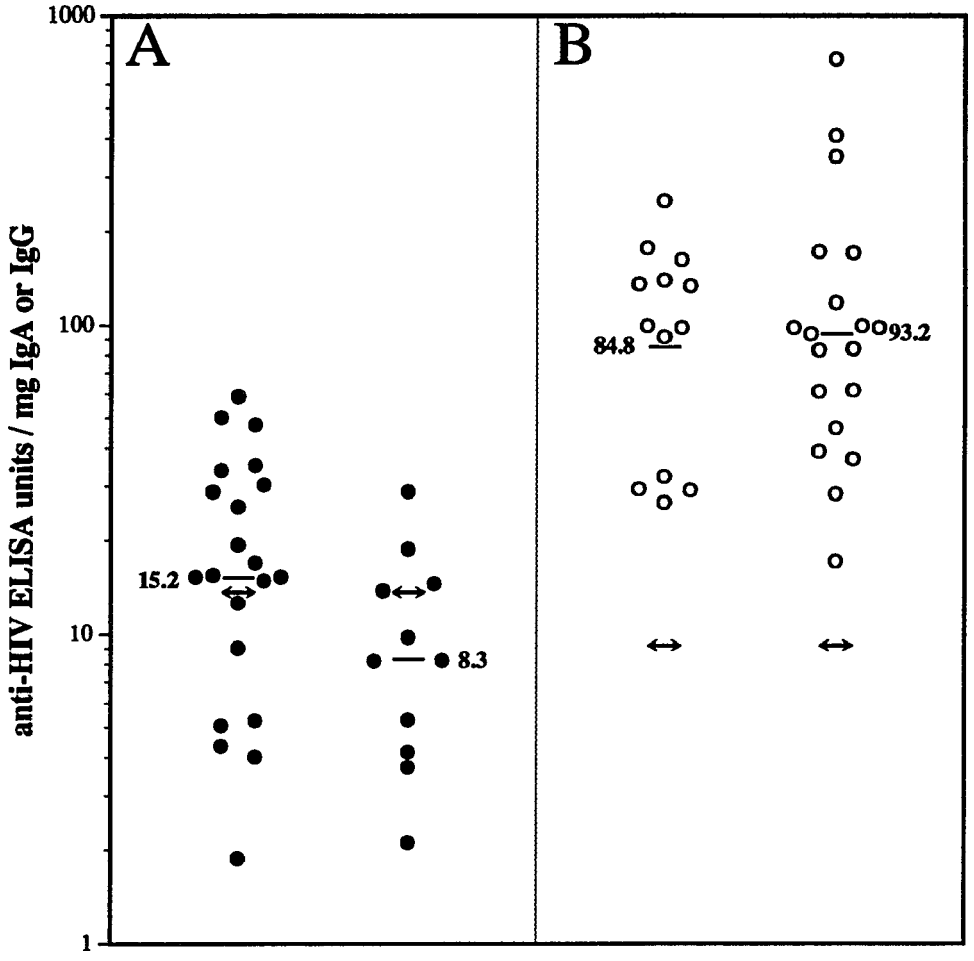

FIG. 4. Proportions of HIV-specific IgA and IgG antibodies in relation to serum hypergammaglobulinemia. Shown are the ELISA units of anti-HIV activity present in (A) IgA samples (●) purified from patients having normal (n = 21) or elevated (n = 11) serum IgA concentrations and (B) IgG samples (o) purified from patients with normal (n = 13) or elevated (n = 19) serum IgG concentrations. Mean anti-HIV ELISA units per milligram for each group are indicated (–) with significance levels (↔). The mean concentration of IgA or IgG present in the sera of individuals comprising each group is shown at the bottom of the graph. Significant differences were observed between the following: normal IgA and elevated IgA, $p = 0.0451$; all IgA and IgG comparisons, $p = 0.0001$.

by IgA of the AIDS group in Fig. 1 appears to be at least partly related to the presence of higher levels of IgA in the sera of these patients. This dilutory phenomenon of HIV antibodies was not observed with IgG purified from individuals with hypergammaglobulinemic IgG. As shown in Fig. 3, elevations of IgG were also present in the sera of more AIDS (70%) than ASY patients (47%). Nevertheless, when patients were separated by levels of serum IgG concentrations, those with elevations were found to contain slightly higher levels of anti-HIV ELISA units per milligram of IgG (Fig. 4). This suggests that although much of the IgG produced by B cells of infected subjects may be in direct response to HIV and dependent on $CD4^+$ T cells, IgA produced is more polyclonal in nature and independent of CD4 status.

IgA and IgG neutralizing capacities

The ability of purified IgA to protect CEM cells from HIV infection was tested in parallel with IgG isolated from 20 seropositive individuals to examine the possibility that the presence of lower proportions of HIV antibodies in IgA samples might also include lower proportions of neutralizing antibodies, rendering IgA less capable of inhibiting infection. As shown in Fig. 5, viral infection could be significantly inhibited by 100μg of IgG purified from 19 of 20 infected subjects. At this concentration however, only 9 of 20 IgA samples were found to inhibit significantly over those of the seronegative control group. In addition, less neutralizing activity was present in IgG and IgA isolated from the sera of individuals with AIDS (Fig. 5A) as compared to that isolated from ASY patients (Fig. 5B). Of the 11 IgG samples purified from ASY patients, 10 inhibited infection by >90%, but only 3 of 9 AIDS IgG samples showed comparable neutralization. Mean inhibition of viral infection, though not statistically different, by IgG for these groups was 94% and 74%, respectively. In contrast, mean inhibition by IgA was 41% for ASY patients and 27% for those with AIDS, with only 2 of 20 IgA samples from infected subjects inhibiting infection >90%. Interestingly, the ability of IgA or IgG to inhibit HIV infection did not correlate with the proportion of total HIV antibodies present in samples.

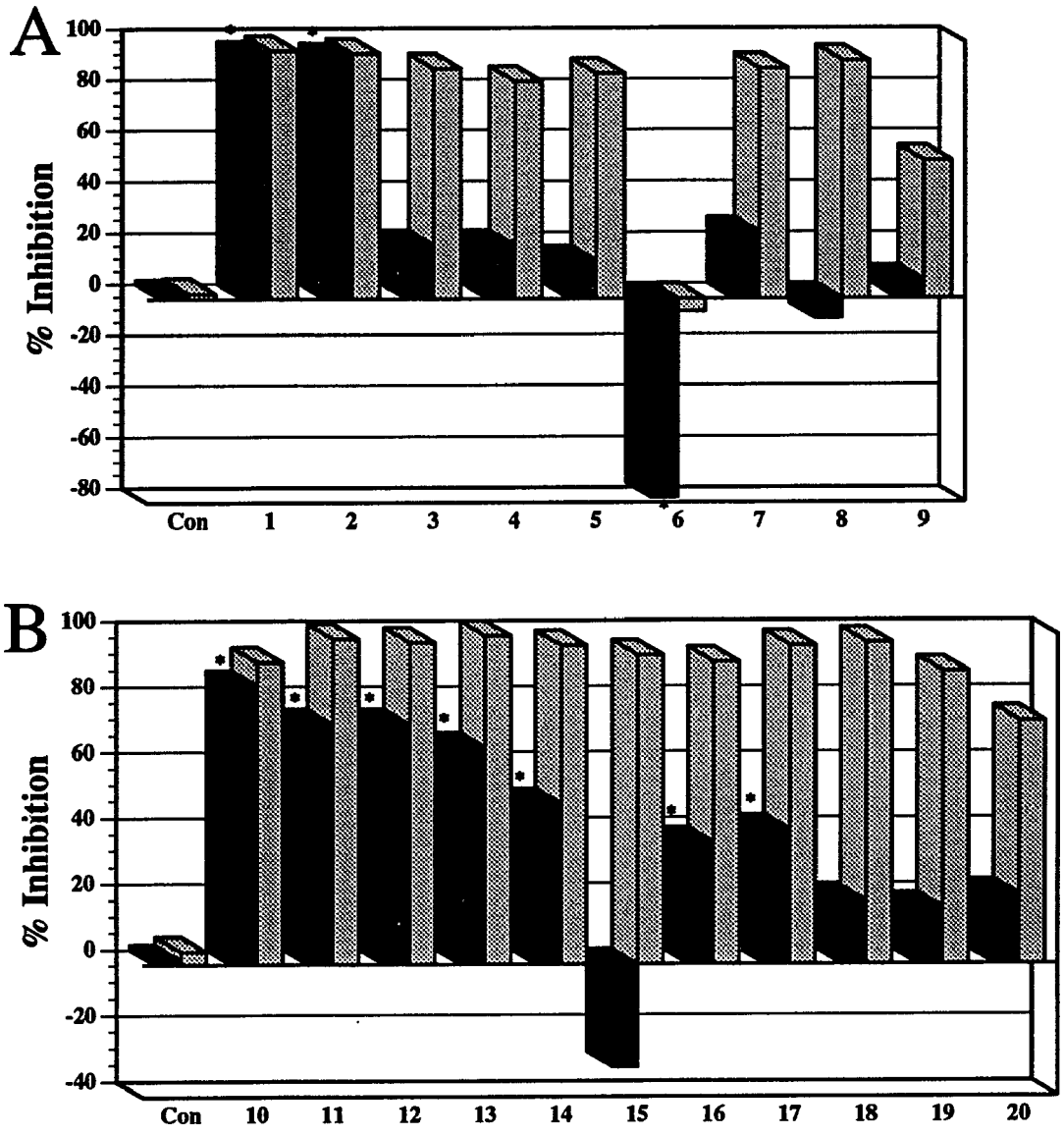

FIG. 5. IgA and IgG neutralizing capacity. One hundred micrograms of IgA1 (black bars) or IgG (gray bars) purified from the serum of (A) 9 AIDS patients and (B) 11 ASY individuals was tested for ability to inhibit HIV infection of CEM cells. Mean inhibition observed by IgA and IgG from six seronegative controls is also shown (Con). With the exception of IgG isolated from patient 6, all IgG samples inhibited significantly over that of controls. Statistically significant inhibition by IgA is denoted by an asterisk. Values on the negative axis represent percentage of viral infection over that observed in control cultures, which received virus alone.

In addition, no correlation of neutralizing activity with CD4 count or serum Ig levels was found. These results do indicate, however, that HIV-infected individuals are capable of producing IgA neutralizing antibodies, although at proportions considerably lower than IgG antibodies present within the total IgG produced.

IgA and IgG anti-V3 antibodies

To determine whether the decreased ability of IgA to inhibit HIV infection observed above may be related to the presence of lower proportions of HIV-1_{IIIB} V3-specific neutralizing antibodies present within this isotype, we examined by ELISA the binding by both IgA and IgG samples to an HIV-1_{IIIB} gp120 V3 peptide (IIIB-V3). Results indicated that disease stage did not influence the reactivity against this peptide. Therefore, in Fig. 6, proportions of IIIB-V3 ELISA units present in IgA and IgG samples from all HIV-seropositive (SP) individuals is shown relative to SN controls. Although IgA from six SP individuals contained significant proportions of anti-IIIB-V3 ELISA units per milligram, specific recognition of the peptide by IgA from the SP group, as a whole, was not observed when statistically compared to background binding by IgA of the SN group. IgG of the SP group did contain significantly higher proportions anti-IIIB-V3 ELISA units per milligram IgG than IgG of the SN group. However, these proportions were not great enough to differ significantly from those present in SP or SN IgA.

Interestingly, the presence of higher proportions of anti-IIIB-V3 IgA or IgG antibodies did not correlate with increased ability of these samples to inhibit infection of CEM cells by the HIV-1_{IIIB} isolate. For example, although IgA purified from patient 1 (see Fig. 5B) had 124.2 anti-IIIB-V3 ELISA units/mg of IgA and inhibited infection by 95%, patient 2 IgA was capable of inhibiting infection by 94% but contained only 14.4 anti-IIIB-V3 ELISA units/mg IgA. Patients 4 and 9 had high proportions of anti-IIIB-V3 ELISA units (87.3 and 88.8, respectively) per milligram IgA that were not capable of significantly inhibiting infection. This suggests that the proportions of IIIB-V3-specific

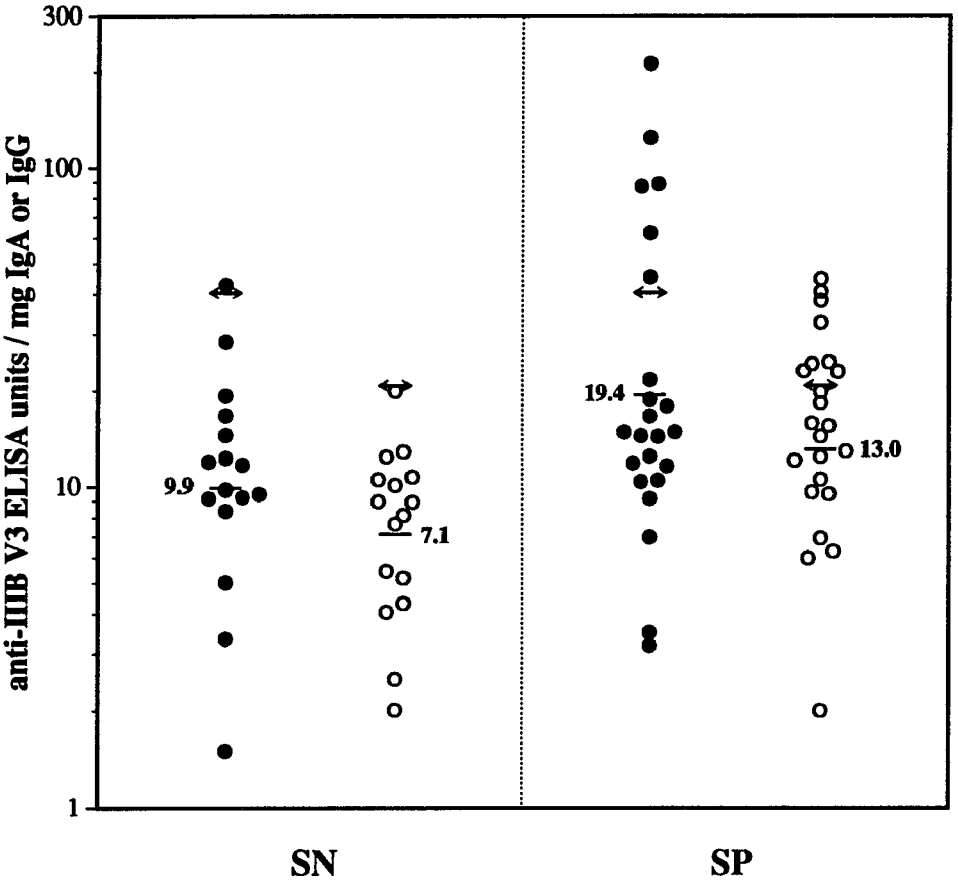

FIG. 6. Proportion of anti-HIV-1IIIB V3-specific antibodies in IgA (●) and IgG (o) purified from 16 SN and 23 SP individuals. Mean levels (–) and the value (↔) at which levels are 3 SD above the mean of SN IgA or IgG are shown. Significant differences were observed only between SN IgG and SP IgG, p = 0.0017.

antibodies in IgA and IgG measured are not high enough to mediate effective neutralization via binding to the V3 loop and that the ability of samples to inhibit HIV-1_{IIIB} infection is likely due to the presence of antibodies directed against other viral determinants.

If IgA V3-specific neutralizing antibodies are produced in infected individuals, detection and quantitation should utilize the V3 loop of a more prevalent isolate. Therefore, IgA and IgG samples were quantitated by ELISA for proportions of antibodies reacting against a peptide (con-V3) containing the gp120 North American consensus V3 loop sequence (Fig. 7). When examined on an individual basis, IgG from both ASY and AIDS patients was found to contain an average of 2.5 times greater proportions of anti-con-V3 ELISA units per milligram purified protein than those present in IgA (not shown). All ASY IgG preparations contained significant (>20.6 ELISA units/mg) proportions of con-V3-specific antibodies with a mean level of 106.8 con-V3 ELISA units/mg of IgG for this group. Reactivity in IgG from AIDS patients was lower at a mean level of 64.2 ELISA units/mg of IgG and fewer patients (88%) in this group displayed IgG binding 3 SD above the mean for IgG of the SN group. Compared to binding by IgA of the SN control group, IgA purified from 80% of ASY and 70% of AIDS patients was found to react significantly with the consensus V3 domain. Although the mean level of con-V3 ELISA units per milligram IgA was again lower for the AIDS group, statistical analysis revealed that, as with IgG, the proportion of con-V3 antibodies present within IgA from the ASY and AIDS group were not significantly different. These results demonstrate that IgA HIV V3-specific neutralizing antibodies are produced by infected individuals, although at proportions lower than those of the IgG isotype.

No preference in binding to either con-V3 or IIIB-V3 was noted between IgA and IgG of the SN group. In addition, no significant associations of IgA and IgG reactivity against either V3 peptides with CD4 counts, proportions of HIV antibodies, serum immunoglobulin concentrations, or neutralization capacity were found.

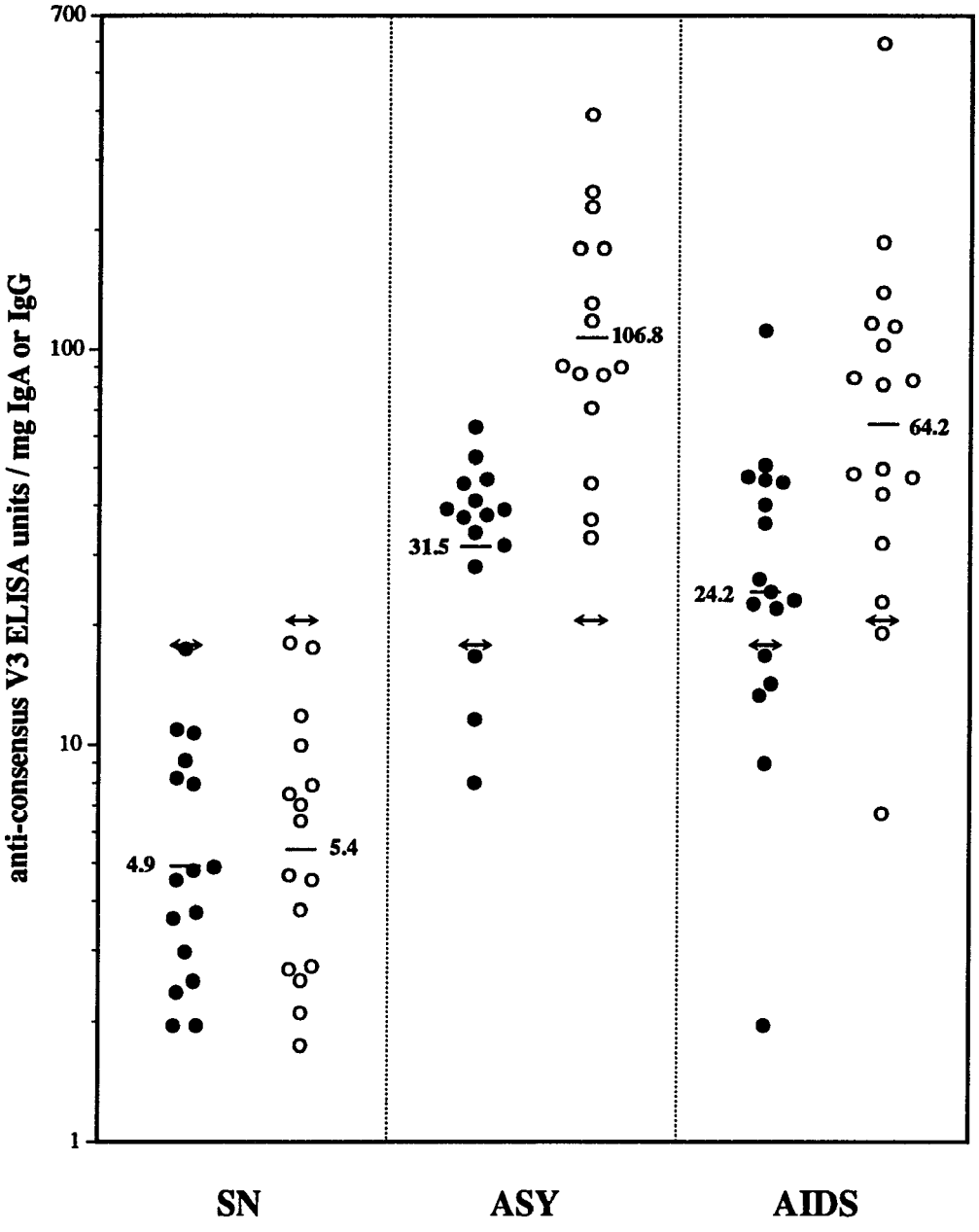

FIG. 7. Proportions of consensus V3 antibodies present in purified IgA (●) and IgG (o) from 16 SN, 15 ASY, and 17 AIDS patients. Mean anti-con-V3 ELISA units per milligram of each protein are shown (–) and arrows (↔) designate values at which levels are 3 SD above that of the mean of the relevant SN group. Statistically significant differences were observed between the following groups: SN IgA and ASY IgA, SN IgG and ASY IgG, SN IgA and AIDS IgA, SN IgG and AIDS IgG, AIDS IgA and ASY IgG, all $p = 0.0001$; ASY IgA and ASY IgG, $p = 0.0002$; AIDS IgA and AIDS IgG, $p = 0.0069$; ASY IgA and AIDS IgG, $p = 0.0097$; AIDS IgA and ASY IgG, $p = 0.0001$.

Discussion

The present findings demonstrate that proportionally fewer IgA HIV- and gp120 V3-specific antibodies constitute the total serum IgA produced by HIV-infected individuals as compared to those proportions present within the IgG produced. When purified IgA and IgG preparations were quantitated on a per milligram basis, infected subjects had, on average, seven-fold and 2.5-fold higher levels of IgG antibodies reacting with HIV and V3, respectively. In light of this, it is not surprising that IgG was more effective than IgA at inhibiting HIV infection *in vitro*. It could be argued that poor neutralization by IgA may be an artifact introduced through our use of the HIV-1_{IIIB} isolate since the majority of seropositive individuals in the U.S. are believed to be infected with isolates more closely approximated by the HIV-1_{MN} strain.[11] However, poor binding of IgA to a V3 loop peptide representing the consensus sequence, to which MN is closely related,[11] was also observed. In addition, the question of why reactivity of IgA and IgG purified from the same individuals contrasts so greatly would still remain.

We initially suspected that decreased responses to HIV may accompany disease progression since IgA and IgG samples purified from AIDS patients contained lower proportions of HIV antibodies than those from ASY subjects. Others have also noted lower serum concentrations of HIV antibodies in subjects with end-stage disease.[12] On closer examination, however, we found that although decreased proportions of HIV-specific IgG antibodies significantly correlated with low CD4 counts of patients, proportions of IgA HIV antibodies did not. Instead, the amount of HIV-specific IgA antibodies present within purified IgA inversely correlated with the concentration of total IgA present in the sera of patients from whom samples were obtained. Individuals with elevated serum IgA, whether ASY or AIDS, had statistically lower proportions of IgA antibodies reacting with HIV. The observed difference between these groups in proportions of IgA HIV-specific antibodies can be explained by the presence of IgA hypergammaglobulinemia in a higher percentage of subjects in the AIDS study

population. Previously we have shown that although parallel increases in both the IgA1 and IgA2 subclasses occur in HIV-infected individuals with IgA hypergammaglobulinemia, HIV antibodies are restricted to the IgA1 subclass.[6] Therefore, the dilutory effect of HIV antibodies within the IgA isotype observed here might be expected to be mediated by increases in nonviral IgA2. However, it is more likely that increases in nonspecific antibodies within both subclasses are responsible for this phenomenon. The reasoning for this is as follows: if all IgA HIV reactivity is restricted to the IgA1 subclass, results can be reexpressed as HIV ELISA units per milligram IgA1 because the concentrations of IgA1 and IgA2 in samples were determined prior to analysis. When the proportion of IgA1 HIV ELISA units was then compared with serum IgA1 concentrations of patients, a statistically significant association of increasing IgA1 with decreasing HIV reactivity was again found. Interestingly, no dilutory effect of IgG HIV antibodies was observed in subjects with IgG hypergammaglobulinemia; proportions of HIV ELISA units per milligram IgG remained equivalent to those of patients with normal serum IgG concentrations.

The dramatic differences we observed in the proportion of HIV-specific antibodies present within the IgA and IgG isotypes is probably partly due to increased numbers of antigen-specific IgG-secreting B cells directed at multiple HIV proteins. IgG generally reacts broadly against all HIV antigens, while IgA almost exclusively binds the envelope glycoproteins, gp120 and gp41.[6] Thus, the greater proportions of HIV-specific IgG antibodies observed in this study may be due to the recognition of more viral determinants present in lysates. However, we also observed predominance of IgG over IgA in binding to the consensus V3 loop. The gp120 disulfide-bridged V3 loop, containing the HIV PND, is characterized by a highly conserved GPG sequence at its tip and hypervariable flanking amino acids.[11] Antibodies generated toward the tripeptide sequence are group-specific since they are capable of neutralizing a wide range of isolates.[13] However, antibodies produced against the PND of one isolate are often unable

to recognize the PND of other isolates and hence are type- or isolate-specific.[13] For instance, HIV-1_{IIIB} and HIV-1_{RF} V3 peptides have been shown to be recognized by only 14 and 22% of infected individuals in North America, presumably because the prevalence of these isolates is relatively uncommon.[11] On the other hand, 65-71% of sera recognized consensus-like V3 peptides, such as that of HIV-1_{MN}.[11] With disulfide-bonded V3 consensus peptides, others have reported reactivity by IgG in the sera of up to 90% of infected Europeans and Africans.[14] In agreement with these results, we observed significant binding to a consensus V3 peptide by purified IgG in 94% of our study population. Although 75% of these subjects also displayed IgA reacting significantly above controls, proportions of anti-con-V3 antibodies in IgA were generally 2.5 fold lower than those present in IgG. Additionally, 95% of individuals examined had IgG capable of inhibiting HIV-1_{IIIB} infection of CEM cells by greater than 50%, but this number dropped to 10% for equivalent amounts of IgA. Poor reactivity with the PND and other gp120 neutralizing epitopes may explain why the neutralization potential for IgA is less than that of IgG. Our results indicate that the ability of IgG and IgA to inhibit HIV-1_{IIIB} infection of CEM cells may be mediated via group-specific determinants, such as the CD4 binding site on gp120,[13] since very little binding to an HIV-1_{IIIB} V3 peptide was observed and reactivity against this peptide did not correlate with the ability to inhibit infection. This suggests that, when present, the levels of anti-HIV-1_{IIIB} V3 antibodies were not sufficient to mediate effective neutralization. Considering the low prevalence of this isolate within the HIV-infected population this is not surprising. It is possible that the neutralization of this isolate was achieved through a synergistic mechanism in the presence of antibodies directed against more than one viral determinant, such as that described for combinations of V3 loop and CD4 binding site antibodies.[15] In addition, other less characterized HIV neutralizing epitopes within gp120 and gp41 have been reported and binding to these regions may also play a role in inhibiting infection.[16,17]

Taken together, our results indicate that IgA responses within HIV-infected individuals markedly differ with those of IgG. Contrasts in proportions of IgA and IgG HIV and V3 antibodies produced may be related to differences in immunoglobulin variable gene usage among IgA and IgG B cell populations. For instance, the V_H3 subgroup is expressed in 70% of human α heavy chains and only 20% of human γ heavy chains.[18] Differences in V_H gene utilization are believed to be determined by and reflect the character of the antigen presented. Murine and human antibodies induced against a variety of antigens have been shown to exhibit restricted patterns of V_H and V_L usage.[3,19] Similarly, analysis of IgG HIV antibodies suggests clonally restricted responses may develop in HIV-infected individuals.[20-22] Coupled with the inability of the humoral immune system to sufficiently respond to mutating HIV epitopes, this has led to the hypothesis that continual cross-stimulation of initially expanded B cell clones by emerging HIV variants may lead to a clonal dominance within the B cell repertoire that is maintained throughout the course of infection.[23] Other B cell populations capable of producing higher affinity antibodies against the same or similar determinant may be suppressed by dominantly established clones.[24] Some evidence that this phenomenon may be associated with HIV infection in humans has been provided;[25] however, it remains to be determined whether IgA antigen-specific responses may be blocked by dominant IgG B cell clones in infected individuals.

In view of the predominant usage of V_H3 by IgA, it may be more relevant that gp120 has been recently shown to directly bind both soluble and membrane Ig expressing this subgroup.[26] This interaction would explain the predominance observed within our seronegative population of IgA over IgG in binding to HIV lysates. The region of gp120 which mediates binding to V_H3 Ig has not yet been elucidated, but is likely to be one which is conserved, since gp120 from different isolates appears to have the same capability.[26] Therefore, the lack of preference we noted between seronegative IgA and IgG in binding to the HIV-1_{IIIB} V3 and consensus V3 peptides, representing a highly

variable region of gp120, would also be consistent with this phenomenon. It may also be the underlying mechanism responsible for the previously reported ability of HIV to directly activate B cells *in vitro*.[27] Stimulated Ig production by V_H3 B cells was observed after culture with gp120.[26] These authors have noted expansion of V_H3-bearing B cells in early clinical stages of HIV infection but deficits of these cells in peripheral blood and lymph node germinal centers of individuals with AIDS.[28] Others have reported depletion of the V_H3 family in anti-HIV antibodies.[21] Because initial stimulation with subsequent deletion are characteristic of T cell superantigens, it has been suggested that gp120 may function as a B cell superantigen.[26] The possibility also cannot be excluded that by virtue of its mitogenic properties for V_H3 B cells, a more marked polyclonal activation of those committed to IgA may occur in HIV-infected individuals and that this phenomenon may be related to the inability to develop higher proportions of HIV-specific IgA antibodies.

Our studies have focused on characterizing responses and function of IgA in the serum of HIV-infected individuals and thus reflect systemic activities. At this time, it is not known whether our findings can be extended to IgA at mucosal sites. Divisions between the systemic and mucosal compartments have been well-documented and are partly illustrated by the differences in levels and origin of Ig present.[29] Serum Ig is derived primarily from the bone marrow and contains higher concentrations of IgG.[30] In contrast, the Ig secreted at mucosal surfaces is locally produced and predominantly IgA.[29] Differences between the systemic and mucosal local microenvironments in which B cells mature are probably responsible for many of the features unique to these regions, including isotype commitment and development of antigen-specific responses. In lieu of the microenvironmental influences and the fact that initial exposure to HIV most often occurs at the mucosa, it seems unlikely that IgA responses in the systemic and mucosal compartments would be comparable. We have had the opportunity to screen only 2 secretory IgA preparations, both derived from colostrum. However, we observed that, as in serum, HIV IgA antibodies in these samples were also restricted to the IgA1 subclass[6]

and proportions of HIV and V3-specific antibodies of the same magnitude as those present in IgA purified from serum (not shown). Evidence for an extension of B cell hyperreactivity to mucosal tissues has also been recently provided.[31] Analogous to serum hypergammaglobulinemia, cervicovaginal fluids obtained from HIV-infected women were found to contain elevated levels of IgG, IgA, and IgM.[31] Similar to our results, IgA antibodies specific for gp160 could be detected in many women but this response was strongly dominated by IgG. Increases in anti-gp160 IgG antibodies were also associated with increases in total IgG at this site. It seems unlikely that passive transudation of serum immunoglobulin may be responsible for these elevations since serum anti-gp160 antibody levels did not correlate with those present in cervicovaginal washes. In addition, because significant numbers of IgG producing B cells are present in the human cervix (P. Crowley-Nowick, personal communication) it seems reasonable that cervicovaginal anti-gp160 IgG antibodies at this site originate through a local antigen-driven response. The low levels of IgA gp160 antibodies with large amounts of nonspecific IgA present in these washes also suggests that similar B cell anomalies occur at this mucosal site. If B cell aberrancies resulting from polyclonal activation are mediated through mitogenic properties of gp120 this may have serious implications in the design of an effective HIV mucosal vaccine. Whether higher titer HIV-specific IgA antibodies can be successfully produced after vaccination with gp160 or gp120 will await human vaccination studies.

Acknowledgments

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HIGH PREVALENCE OF IgA HIV-1 INFECTION-ENHANCING ANTIBODIES IN SERUM OF HIV-INFECTED INDIVIDUALS AND MASKING BY IgG

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Abstract

IgA and IgG purified from sera of 20 HIV-infected individuals were separately examined for ability to enhance HIV-1 infection of the U937 cell line. Both isotypes were capable of mediating enhanced infection of these cells. IgA-mediated antibody-dependent enhancement (ADE) of HIV infection could be inhibited by preincubation of U937 cells with a monoclonal antibody specific for the Fcα receptor. Within this study population, IgA from twice as many individuals (14/20) exhibited infection enhancing activity as compared to IgG. Further, IgA-mediated ADE was most prevalent in asymptomatic HIV-seropositive subjects (9/9). Enhanced HIV infection of U937 cells by IgA was most often observed at concentrations of 5μg/ml and 0.5μg/ml, equivalent to serum dilutions in the range 10^{-3} to 10^{-5}. In contrast, concentrations of IgG mediating ADE (0.5μg/ml and 0.05μg/ml) were generally present in sera at dilutions from 10^{-4} to 10^{-6}. Greater concentrations of IgG had no enhancing effect or were able to inhibit HIV infection of cells. After adjusting IgG concentrations to those equivalent to serum IgG and combining with enhancing concentrations of IgA, IgA-mediated enhancement was not observed unless IgG was present at a concentration which also exhibited enhancement. These results suggest that, in comparison to IgG, HIV-infected individuals may more often produce IgA antibodies which recognize enhancing determinants of HIV. IgA-mediated ADE of HIV infection may not play a significant role in systemic HIV pathogenesis due to the presence of higher concentrations of IgG, which also contains a greater proportion of HIV-specific antibodies. However, the production of such IgA HIV-enhancing antibodies at mucosal sites, where very few IgG plasma cells are present, could largely contribute to the pathogenesis of HIV infection and mucosal aberrancies associated with this disease. In addition, the ability of IgA to mediate enhancement could greatly interfere with the development of HIV vaccines designed specifically to induce HIV IgA antibodies at mucosal surfaces.

Introduction

The ongoing effort to develop a vaccine that will effectively protect the world population from HIV-1 is becoming more imperative as the epidemic continues to grow. However, despite some limited success in the vaccination of chimpanzees against HIV-1 (1), recent vaccine trials in humans have been extremely discouraging (2). It has been speculated that vaccine failure may be related to an inability to induce production of neutralizing antibodies in vaccine recipients which sufficiently cross-react with the HIV-1 principal neutralizing determinant (PND) of primary field isolates. In view of the enormous diversity that arises within the envelope genes of HIV-1 (3), particularly the third hypervariable (V3) domain of gp120 which contains the PND (4), this is a reasonable assumption. However, it is also conceivable that both the inability to protect vaccinees and the failure of the immune system to eradicate virus in those individuals already infected could be related to the development of antibodies which may pathologically contribute to this chronic infection through mediating enhanced proliferation and dissemination of HIV-1.

Antibody-dependent enhancement (ADE) of HIV-1 infection has been observed *in vitro* with many different HIV-1 isolates of both T cell- and monocyte-tropic phenotypes, using target cells which express CD4 and Fcγ receptors (FcγR) such as the U937 promyelomonocytic cell line, PHA-stimulated PBMC, and primary macrophage cultures (5-12). The ability of heat-inactivated HIV-seropositive sera or IgG purified from such sera to accelerate or augment production of HIV by these cells has been clearly demonstrated by many groups (5-12). In addition, although sera from HIV-infected individuals often exhibit poor V3-directed "isolate- or type-specific" neutralizing activity against homotypic isolates (13), enhanced infection of PBMC or macrophages by homotypic isolates in the presence of autologous serum can be demonstrated (12). It has been suggested that ADE may contribute to the pathogenesis of HIV infection *in vivo*

since enhancing activity in sera for homotypic HIV strains increases throughout the course of disease and is most prevalent in patients with AIDS (12).

A similar phenomenon, termed complement-mediated ADE (C-ADE), has also been observed with HIV antibodies in the presence of a source of active human complement. C-ADE activity in seropositive sera has been demonstrated with HIV-1_{IIIB} using EBV-transformed B cells and the MT-2 and MT-4 T cell lines, all of which co-express CD4 and the CR2 complement receptor, necessary for demonstrating C-ADE (14-17). In regard to HIV pathogenesis, one of the most important findings with respect to C-ADE of HIV infection has been the observation that the presence of enhancing antibodies and complement leads to a marked reduction in the capacity of sera to neutralize HIV infection (14,15).

Although all FcγR have been implicated as potential participants in IgG-mediated ADE (5-8), the possibility that anti-HIV IgA antibodies and the FcαR could similarly participate in ADE of HIV infection has not been directly examined. As with IgA in serum, secretory IgA (S-IgA) in saliva and cervical secretions of HIV-infected individuals predominantly reacts against the HIV-1 envelope glycoproteins (18-20), on which enhancing epitopes are likely (21,22). We have recently shown that IgA purified from the sera of infected individuals can inhibit HIV-1_{IIIB} infection of T cells *in vitro*; however, it mediates neutralization of this isolate via viral determinants other than the V3 loop and has a weaker capacity for neutralization when compared to the same concentration of IgG (23). As expected, we observed that IgA reacts more strongly with a V3 loop peptide of North American consensus sequence than the HIV-1_{IIIB} V3 loop. However, quantitative analyses of these IgA and IgG samples demonstrated that the proportion of anti-consensus V3 antibodies present within the IgG isotype were, on average, 2.5-fold greater than those present in an equivalent amount of IgA (23). Similarly, we found that the proportion of IgG HIV-specific antibodies generally

averaged seven-fold greater than the proportion of specific antibodies within the IgA isotype (23).

Considering these data and the fact that seropositive sera often contain 4- to 6-fold greater concentrations of IgG than IgA (23), it is not surprising that an accurate depiction of IgA HIV antibodies present in such sera can be difficult to achieve using typical western blot techniques unless IgG is removed (24). For the same reason, the presence of HIV infection-enhancing IgA antibodies that are not readily apparent, due to greater concentrations of HIV-specific IgG which may mediate neutralization or simply bind and block viral determinants potentially recognizable by IgA, is possible. Although enhancement by IgA could be blocked by IgG in serum, it is highly unlikely that this would occur at mucosal sites where IgA-secreting plasma cells generally predominate, and concentrations of this isotype are often the greatest (reviewed in Ref. 25). Therefore, an ability of IgA to mediate ADE could be devastating to the development of vaccines designed to elicit protective HIV antibodies at mucosal surfaces, such as those in the male and female genital tracts. In addition, it is clear that within individuals already infected, mucosal mononuclear cells harbor HIV (26); even two-fold increases in virus proliferation could greatly contribute to the pathogenesis of HIV infection considering both the enormous area encompassed by the mucosa and the prolonged course of this disease.

In an effort to determine whether IgA may enhance HIV infection, we have chosen to first study this phenomenon using IgA from the systemic compartment, since we have previously identified many HIV-associated characteristics of serum IgA. To avoid possible interference by IgG, IgA and IgG were affinity-purified from the serum of HIV-infected individuals and each preparation examined separately for ability to enhance HIV-1_{IIIB} infection of the $CD4^+$, $Fc\gamma R^+$, $Fc\alpha R^+$ U937 cell line. The purpose of these experiments was to determine whether HIV-infected individuals may produce IgA antibodies capable of enhancing HIV infection and, if so, to establish conditions under

which this phenomenon could be further investigated using S-IgA from infected or immunized individuals.

Materials and Methods

Study subjects

Serum from 20 HIV-seropositive (SP) study participants was obtained from the UAB Center for AIDS Research Clinical Core Facility and stored at -70°C until use. These individuals were clinically classified in accordance with the CDC guidelines established in February 1993. Nine were asymptomatic and accordingly placed in category A, 4 had evidence of opportunistic infections characteristic of category B, and 7 were diagnosed with clinical conditions listed in the AIDS surveillance case definition for category C. The breakdown of these individuals within each category in regard to CD4 counts is as follows: those having 500 cells/mm^3 or greater are denoted by a 1, 200-499 cells/mm^3 by a 2, and <200 cell/mm^3 by a 3. Serum was also voluntarily contributed by 10 healthy HIV-seronegative (SN) individuals who represented the control group.

Purification of IgA and IgG

IgA and IgG were affinity purified as previously described (18). Briefly, removal of IgG from sera was accomplished with Protein G Sepharose (Pharmacia, Piscataway, NJ), while IgA was isolated on a column of immobilized monoclonal reagent with specificity for human IgA1 and IgA2. Eluted IgG and IgA were dialyzed in PBS, 0.22μm sterile-filtered, and analyzed for Ig content by ELISA. Purity was assessed at 99-100% for IgG and 94-99% for IgA.

ELISA assay for IgA and IgG

IgA or IgG present in purified samples or in sera of study subjects was measured as described (23). Briefly, 96 well microtiter plates (Dynatech, Chantilly, VA) coated overnight with reagents specific for human IgG (Rockland, Gilbertsville, PA), IgA1, or IgA2 (kindly provided by Dr. J. Radl, IVVO-TNO, Leiden, The Netherlands) were blocked with PBS containing 5% horse serum (GIBCO, Gaithersburg, MD) and 0.05%

Tween-20 and then reacted with dilutions of either purified Ig or sera, followed by treatment with biotinylated affinity-purified goat $(Fab')_2$ fragments specific for IgG or IgA (Tago, Burlingame, CA). IgG concentrations were determined after consecutive treatments with avidin-labeled peroxidase (Sigma, St. Louis, MO) and H_2O_2 in 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) enzyme substrate and then measurement of absorbance values at 414 nm in an automated plate reader (Anthos Reader 2001, Bioquip, Atlanta, GA). IgA1 and IgA2 concentrations were determined following development with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA), phosphatase substrate buffer (Sigma), and measurement of absorbance values at 405 nm. Sample concentrations were determined in relation to simultaneously assayed Ig reference standards using calibration curves constructed by a computer program based on 4-parameter logistic algorithms. IgA1 and IgA2 concentrations were combined to obtain the total IgA concentration.

Cells and virus

The U937 promyelomonocytic cell line (American Type Culture Collection, Rockville, MD) was maintained in culture with RPMI 1640 (GIBCO) supplemented with 10% FBS (Hyclone Laboratories, Logan, UT), 0.3mg/ml L-glutamine, 100 units/ml penicillin, and 0.1mg/ml streptomycin (all GIBCO). These cells were routinely monitored by DNA fluorography methods (27) for mycoplasma infection. Only cells determined to be mycoplasma negative were used in experiments. For enhancement assays, a virus stock was generated as previously detailed (23) after harvesting medium containing virions produced within a 24 h period by HIV-1_{IIIB}-infected U937 cells. Medium with virus was 0.45μm sterile filtered, aliquotted, and frozen at -70°C. As previously described (23), this viral stock was found to contain 1 x 10^5 $TCID_{50}$ /ml by 21 d endpoint titration assay with U937 cells.

Immunofluorescence

All procedures were performed at 4°C with 1 x 10^6 U937 cells which had been washed in a buffer of PBS containing 1% BSA and 0.1% NaN_3. To determine FcαR expression, cells were incubated for 30 min with 0.5μg/ml of the anti-FcαR mAb My43 (28) or an equivalent concentration of MOPC-5.3μ mAb, an isotype-match control mouse IgM with specificity for dinitrophenyl (kindly provided by Dr. J. Mestecky, UAB). Cells were similarly incubated for 30 min with 0.5μg/ml Leu3a (Becton Dickinson, San Diego, CA) or an irrelevant isotype-match mouse IgG1 for analysis of CD4 expression. For studies examining the binding of IgA or IgG to U937, equivalent amounts of purified IgA or IgG were pooled from 10 SN individuals, adjusted to desired concentration, and incubated with cells for 45 min. Following incubation with the above reagents, cells were washed twice in buffer and reacted with FITC-labeled $F(ab')_2$ goat anti-mouse Ig, anti-human IgA, or anti-human IgG (Southern Biotechnology Associates, Birmingham, AL). After 30 min, U937 cells were washed three times and fixed in 1% paraformaldehyde, and cell-associated fluorescence was determined using a FACStar (Becton Dickinson).

Reverse transcriptase assay

Viral production was monitored by measuring reverse transcriptase (RT) activity present in cell-free culture medium as described (23). Briefly, 15μl of thawed medium from each culture was mixed in reaction cocktail to yield a final volume of 40mM Tris, 32 mM $MgCl_2$, 40mM DTT, 200mM EGTA, 2% Triton X-100, 2.5μCi [^{3}H]-dTTP (NEN Research Products, Atlanta, GA), and 80μg/ml poly(rA)·$p(dT)_{12-18}$ (Pharmacia) at pH 7.6. After 2 h at 37°C, 22μl of each reaction mixture was transferred to a designated DE81 ion exchange chromatography paper square (Whatman, Hillsboro, OR) and allowed to dry at room temperature. Paper squares were washed six times in 5% (w/v) Na_2HPO_4, twice in dH_20, and twice in 70% ethanol to remove nonspecific material prior to immersion in EconoSafe counting cocktail (Research Products International, Mount

Prospect, IL). Radioactivity associated with each square was then determined in a scintillation counter.

Enhancement assay

Sterile IgA or IgG samples purified from sera of SP or SN individuals were placed in 5ml sterile tissue culture tubes (Becton Dickinson) and adjusted with DPBS to yield desired concentration in a final volume of 100µl. Tubes designated as cell controls received 100µl of DPBS and 100µl of RPMI 1640 medium, while those for virus controls received 100µl of DPBS alone. All samples were assayed in duplicate. Virus (100µl containing 5000 $TCID_{50}$ diluted in medium) was then added to all tubes, except cell controls, and incubated at 37°C for 1 h, after which time, 5 x 10^5 U937 cells in 100µl were added to each tube and incubation was resumed at 37°C. Tubes were shaken at 15 min intervals throughout both incubation periods to facilitate interaction of contents. Cells were then washed twice in 4 ml DPBS, resuspended in 1 ml of media, transferred to 24 well plates, and cultured at 37°C in a 5% CO_2 humidified atmosphere. Every 72 h, cultures were split 1:5 by removing 200µl of each cell suspension and placing in another well containing 800µl fresh medium. Remaining culture medium was depleted of cells through low-speed centrifugation and stored at -20°C until assayed for RT activity.

To determine whether samples enhanced HIV infection, an enhancement index (EI) value was determined from the RT activity present in culture medium 6 d after initiating experiments. To calculate the EI, RT activity was first averaged for duplicate cultures. These values were then adjusted for background by subtracting RT activity in cell controls (generally 200 cpm/15µl culture medium). RT values for each IgA or IgG culture were next divided by the RT activity of virus control cultures to obtain the EI. Using this method to express results, EI values >1.0 indicate increased viral production, while values <1.0 represent inhibition of infection.

Blocking assays were performed identically with the exception that prior to addition to tubes containing IgA samples and virus, U937 cells were incubated on ice for

30 min with either 0.5μg/ml of My43 or MOPC-5.3μ mAb. For these experiments, the EI for IgA samples was determined in relation to RT activity present in virus control cultures containing cells which had been preincubated with the same mAb.

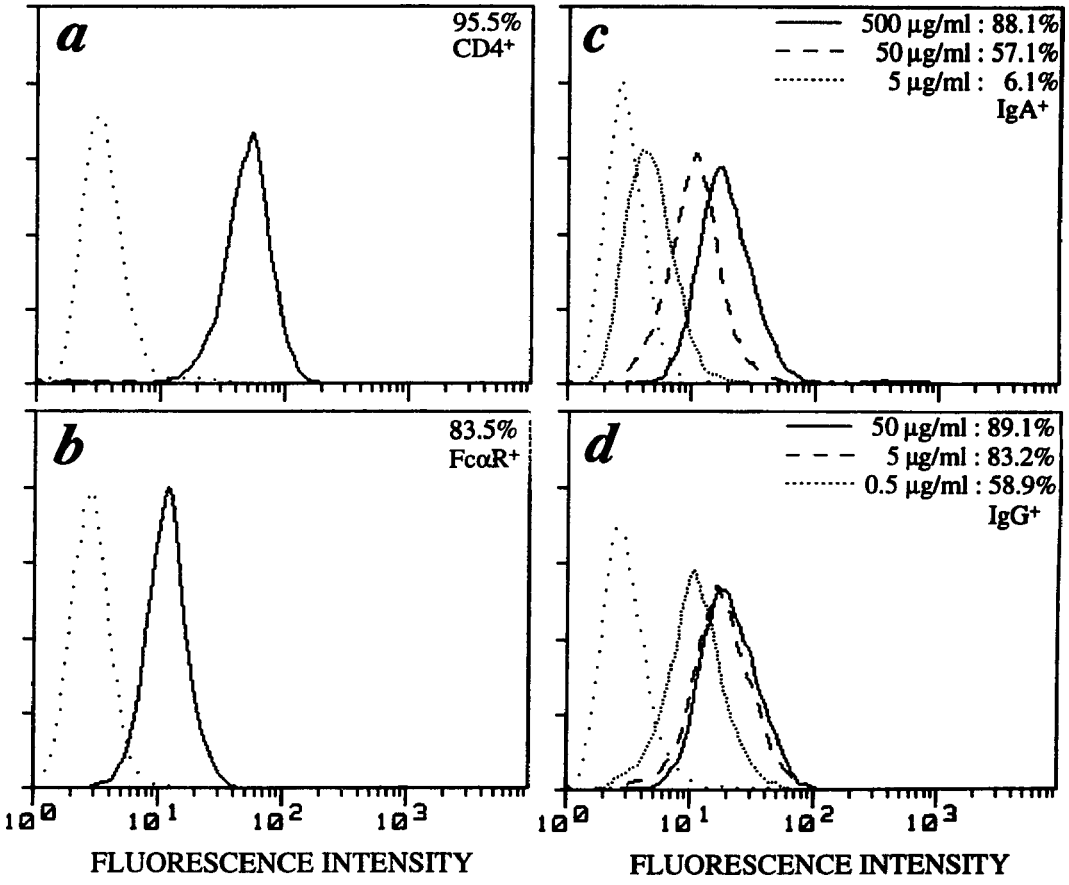
Results

FcαR expression and binding of IgA and IgG to U937

Because the phenotype of U937 cells appears to vary among laboratories, flow cytometric experiments were initially performed to establish some relevant phenotypic characteristics of this cell line, particularly their capability for binding IgA. As shown in Fig. 1a, 95.5% of U937 express high levels of CD4. Immunofluorescence analysis using the previously characterized My43 mAb (28) indicated that 83.5% of these cells display the FcαR (Fig. 1b). In close agreement, 88.1% of cells were found to express IgA on their surface following incubation with a saturating concentration (500μg/ml) of pooled SN IgA (Fig. 1c). Numbers of cells with IgA bound rapidly decreased with decreasing IgA concentrations; only 57.1% and 6.1% surface-IgA positive cells could be detected by immunofluorescence following incubation with 50μg/ml and 5μg/ml of SN IgA, respectively (Fig. 1c). No significant differences were observed in the binding to U937 by this pooled SN IgA preparation and separate preparations of IgA purified from 5 SP individuals (not shown).

In contrast to results with IgA, considerably lower concentrations of SN IgG could be used to demonstrate binding of IgG to U937 cells (Fig. 1d). Following incubation with 0.5μg/ml, IgG could be detected on the surface of 58.9% of cells. Binding of this non-aggregated IgG by U937 is presumably mediated through FcRI, the high affinity Fcγ receptor, although these cells have also been reported to express the low affinity, FcRII IgG receptor, which binds aggregated IgG or IgG immune complexes (29). Larger concentrations of IgA may be required to demonstrate binding to U937 because the FcαR has a lower affinity for IgA compared to the affinity of FcRI for IgG (29,30).

FIGURE 1. Phenotypic analysis of U937 cells. Expression of a) CD4 and b) FcαR were determined using Leu3a and My43 (solid lines), respectively, isotype-matched control reagents (dotted lines), and FITC-labeled anti-mouse Ig antibody. Cells reacted with varying concentrations of c) SN IgA or d) SN IgG were stained, respectively, with anti-human IgA or anti-human IgG antibodies conjugated to FITC, as described in Materials and Methods. The lightly dotted lines on the far left represent profiles obtained after reacting cells with the latter reagents only. Vertical axes correspond to relative numbers of cells. Results shown are representative of one of two separate experiments.


a
95.5%
CD4+
b
83.5%
FcαR+
c
500 µg/ml : 88.1%
50 µg/ml : 57.1%
5 µg/ml : 6.1%
IgA+
d
50 µg/ml : 89.1%
5 µg/ml : 83.2%
0.5 µg/ml : 58.9%
IgG+
10^0
10^1
10^2
10^3
FLUORESCENCE INTENSITY
FLUORESCENCE INTENSITY

The My43 mAb has been previously demonstrated to block binding of IgA but not IgG to peripheral blood monocytes, neutrophils, and the HL-60 myelomonocytic cell line (28,31). In separate experiments, prior incubation of U937 cells for 30 min with 0.5μg/ml My43 mAb, followed by washing and subsequent incubation with either 500μg/ml SN IgA or 50μg/ml SN IgG, was similarly found to inhibit binding of IgA but not IgG to these cells; although 85.4% U937 cells had detectable IgG, only 7.3% surface-positive IgA cells could be detected by immunofluorescence (not shown).

Effects of SN IgA or SN IgG on HIV-1 production

Incubation of U937 with heat-aggregated normal human γ-globulin in the presence of HIV has been previously observed by others to slightly augment levels of virus produced in this cell line (32). We similarly noted in preliminary enhancement assays that RT activity was greater in culture media of U937 cells which had been exposed simultaneously to HIV and certain concentrations of control SN Ig samples than in those exposed to virus alone. This suggested that IgA and IgG might nonspecifically stimulate U937 cells. Therefore, prior to assaying SP Ig samples for enhancement, background EI values were established using IgA and IgG from a total of 10 SN individuals.

As shown in Table 1, levels of HIV in cultures of cells which had been incubated with high concentrations of SN IgA (500μg/ml and 50μg/ml) and HIV for 1 h averaged 1.48- and 1.43-fold, respectively, over those in virus control cultures. With lower IgA concentrations, this effect was minimal or not observed. Greater levels of HIV were also found when cells were similarly exposed to SN IgG and virus. However, unlike IgA, much lower concentrations of SN IgG could augment viral production; purified samples of this isotype were still capable of increasing levels of HIV an average of 1.16-fold and 1.11-fold over those in control cultures at the lowest concentrations (0.5μg/ml and 0.05μg/ml, respectively) tested.

Table 1. Effect of SN IgA and IgG on HIV infection in U937 cells[a]

	IgA (μg/ml)					IgG (μg/ml)				
SN	500	50.0	5.00	0.50	0.05	500	50.0	5.00	0.50	0.05
1	1.16	1.16	1.20	0.96	1.00	1.03	1.09	1.36	1.09	1.08
2	1.52	1.58	1.15	1.13	0.98	1.06	1.26	1.30	1.26	1.05
3	2.34	1.68	1.42	1.08	1.00	1.18	1.25	1.34	1.16	0.98
4	1.26	1.22	1.12	1.24	1.16	1.34	1.18	1.46	1.10	1.16
5	1.18	1.05	0.92	0.96	1.00	1.04	1.18	1.65	1.13	1.25
6	1.65	1.42	1.17	1.00	1.10	1.09	1.11	1.09	0.99	0.96
7	1.37	1.28	1.06	1.04	1.00	1.44	1.50	1.70	1.31	1.17
8	1.45	1.75	1.12	0.92	0.97	1.10	1.13	1.08	1.23	1.22
9	1.06	1.26	1.01	0.90	0.94	1.48	1.75	1.23	1.27	1.02
10	1.80	1.91	0.99	0.92	1.08	1.34	0.91	0.94	1.06	1.18
mean[b]	1.48	1.43	1.12	1.02	1.02	1.21	1.27	1.32	1.16	1.11
SD	0.38	0.28	0.14	0.11	0.07	0.17	0.25	0.24	0.10	0.10
EI*[c]	2.62	2.27	1.54	1.35	1.23	1.72	2.02	2.04	1.46	1.41

[a]Indicated concentrations of IgA and IgG purified from sera of 10 SN individuals were incubated for 1 h with HIV. U937 cells were next added and incubation continued for another h. Cells were then washed and placed in culture, as described in Materials and Methods. Results shown represent the average EI determined from RT activity present in culture medium 6 d after initiating three separate experiments.

[b]The mean EI was calculated for each IgA and IgG concentration and is shown with the SD immediately below.

[c]EI values, three standard deviations above the mean EI for each concentration, at or over which enhancement of HIV infection is considered statistically significant in subsequent analyses with SP IgA or IgG.

Increased production of virus by U937 incubated with non-HIV antibodies is probably related to binding by Ig to FcR. Concentrations of IgA or IgG which were found to augment viral production correlate well with concentrations at which both of these isotypes could be detected on cell surfaces by immunofluorescence (Fig. 1). Increased viral production does not appear to be related to increased cellular proliferation since no significant differences were observed in total numbers or viability of cells in any cell cultures on days 3 and 6 when examined by Trypan blue exclusion (not shown).

Enhancement of HIV infection by SP IgA and IgG

IgA and IgG purified from sera of 20 SP study subjects were next tested for ability to enhance HIV infection in U937 cells. Enhancement was considered significant only if the EI obtained from averaging results of 3 separate experiments was 3 SD over the mean EI value for SN control samples at the identical concentration (see Table 1). Of the 20 SP IgG samples examined, 7 (35%) enhanced HIV production significantly above that of SN controls at concentrations ranging from 0.05-5μg/ml (Table 2). The serum dilutions which would contain those concentrations of IgG found to enhance were determined to range from 10^{-4} to 10^{-6} but were most often observed at 10^{-5} (Table 2).

Data for those SP IgA found to significantly enhance HIV infection are presented in Table 3. Of the total SP IgA samples examined, 14/20 (70%) significantly enhanced HIV infection at one or more of the concentrations tested (Table 3). Enhancement by IgA was most often observed with sample concentrations of 5μg/ml, or equivalent serum dilutions of 10^{-3} or 10^{-4}, 10- to 100-fold lower than those for IgG. The magnitude of enhancement by SP IgA was somewhat greater than that found for IgG. EI values ranging from 2.34 to 3.62 were often obtained with SP IgA. After subtraction of the appropriate control SN IgA mean EI, these values were found to correlate with 1.22- to 2.5-fold increases in viral production. However, the highest EI obtained with SP IgG was 2.52, or 1.2 when adjusted in comparison to the EI for SN IgG at this concentration. Also in contrast with SP IgG, very little or no inhibition of HIV infection was observed with

Table 2. Effect of SP IgG on HIV infection in U937 cells[a]

SP	IgG (μg/ml) 500	50.0	5.00	0.50	0.05	equivalent serum dilution[b]
1	0.53 ± 0.05	1.55 ± 0.12	1.79 ± 0.18	1.36 ± 0.10	1.60 ± 0.07*	4.8×10^{-6}
3	0.70 ± 0.07	1.15 ± 0.16	1.41 ± 0.13	1.99 ± 0.11*	1.59 ± 0.11*	2.0×10^{-5}; 10^{-6}
4	0.13 ± 0.08	1.60 ± 0.10	2.52 ± 0.14*	1.46 ± 0.08*	1.38 ± 0.09	2.4×10^{-4}; 10^{-5}
5	1.14 ± 0.11	1.89 ± 0.13	2.32 ± 0.10*	1.46 ± 0.14*	1.27 ± 0.13	5.6×10^{-4}; 10^{-5}
10	1.06 ± 0.12	1.02 ± 0.12	1.34 ± 0.16	1.62 ± 0.09*	0.98 ± 0.10	2.2×10^{-5}
15	0.36 ± 0.04	0.74 ± 0.09	2.18 ± 0.11*	1.40 ± 0.12*	1.11 ± 0.12	2.9×10^{-5}; 10^{-6}
20	0.49 ± 0.08	1.22 ± 0.14	2.05 ± 0.15*	1.12 ± 0.13	1.08 ± 0.08	2.7×10^{-5}

[a]IgG purified from sera of 20 SP individuals was examined at the concentrations indicated for ability to enhance HIV infection of U937 cells as described in Materials and Methods. Results represent the average EI ± S D determined from three separate experiments and are shown only for those IgG samples which exhibited enhancement. Significant EI values (see Table 1) are denoted by an asterisk.

[b]The dilution of the SP individual's serum containing an IgG concentration equivalent to the concentration of purified IgG which significantly enhanced HIV infection was calculated after determining IgG concentration in the serum by ELISA.

Table 3. IgA-mediated enhancement of HIV infection[a]

SP	IgA (μg/ml)	EI	equivalent serum dilution[b]	serum IgG conc. (μg/ml)[c]
1	5.0	3.62 ± 0.13	4.1×10^{-3}	44.2
2	5.0	1.94 ± 0.09	3.3×10^{-3}	90.0
3	0.5	1.96 ± 0.10	1.0×10^{-3}	25.6
	0.05	1.59 ± 0.14	1.0×10^{-4}	2.5
4	5.0	1.66 ± 0.07	1.8×10^{-3}	37.3
5	5.0	1.74 ± 0.11	2.6×10^{-3}	70.7
	0.5	1.41 ± 0.15	2.6×10^{-4}	7.0
6	0.5	1.48 ± 0.08	1.4×10^{-4}	15.2
7	5.0	2.21 ± 0.13	7.0×10^{-3}	94.3
	0.5	1.63 ± 0.09	7.0×10^{-4}	9.4
8	5.0	2.37 ± 0.12	3.7×10^{-3}	76.6
9	5.0	2.34 ± 0.16	8.1×10^{-4}	8.1
11	5.0	1.66 ± 0.09	2.9×10^{-3}	70.1
12	50.0	2.73 ± 0.06	1.0×10^{-2}	400.4
13	0.5	1.65 ± 0.13	1.3×10^{-3}	51.4
15	0.5	2.71 ± 0.16	2.4×10^{-4}	4.2
20	5.0	3.06 ± 0.15	3.2×10^{-4}	6.0
	0.5	1.68 ± 0.10	3.2×10^{-5}	0.6

[a]IgA purified from sera of 20 HIV-infected individuals was tested at 10-fold concentrations, ranging from 0.05-500μg/ml for ability to enhance HIV infection of U937 as described in Materials and Methods. The average EI ± SD, determined from three separate experiments, is shown only for those IgA concentrations found to significantly enhance infection (see Table 1).

[b]The serum dilution containing a concentration of IgA equivalent to the sample concentration of IgA which yielded a significant EI was calculated after determining total serum IgA concentration of study subjects by ELISA.

[c]The IgG concentration present in the individual's serum at the indicated dilution was calculated from total serum IgG concentration, determined by ELISA.

500μg/ml of SP IgA. Results determined from three separate experiments with all 20 SP study subjects yielded a mean EI at this concentration of 1.15 for SP IgA and 0.64 for SP IgG (not shown).

Unlike SN Ig samples, the ability of SP IgA and SP IgG to enhance HIV infection in this cell line does not appear to be solely related to stimulatory effects of IgA and IgG exerted on binding to FcR. First, as can be seen in Tables 1 and 2, SP IgA and IgG were both found to significantly enhance HIV infection at varying concentrations, particularly lower concentrations at which effects of SN IgA or IgG were minimal on HIV production. Second, HIV infection was not significantly enhanced when experiments were performed such that the 1 h incubation period of virus and Ig was excluded and cells, SP Ig samples, and virus were instead all combined simultaneously (not shown). When the enhancement assay was again modified such that cells were incubated first with IgA or IgG for 1 h, washed twice and then exposed to HIV for another hour, again, no significant enhancement was observed with SP Ig samples (not shown). Under these latter conditions, however, levels of HIV present in cultures of cells initially exposed to high concentrations (500μg/ml and 50μg/ml) of SN IgA and IgG were almost identical to those observed in standard enhancement assays, yielding EI values similar to those shown in Table 1. This suggests that the consequences of nonspecific stimulation of U937 cells by very high concentrations of IgA or IgG are conducive to HIV infection. As will be shown shortly, in the case of IgA, this stimulatory effect appears to involve binding to the FcαR.

In Table 4 results from enhancement assays are summarized for all HIV-infected individuals participating in this study in relation to clinical stage of disease and CD4 count. In agreement with data of others who have used the U937 cell line and the HIV-1_{IIIB} isolate (7), no correlation between disease stage or CD4 count and the presence of IgG mediating ADE of HIV infection was apparent in these subjects. However, IgA purified from serum of 100% (9/9) of these asymptomatic individuals exhibited HIV

Table 4. Relationship of disease stage and CD4 count to ADE of HIV infection[a]

SP	CDC Stage	CD4 cells per mm^3	Enhancement[b]	
			IgA	IgG
1	A1	950	+	+
2	A1	901	+	-
3	A1	640	+	+
4	A1	519	+	+
5	A1	570	+	+
6	A2	357	+	-
7	A2	242	+	-
8	A3	181	+	-
9	A3	153	+	-
10	B3	118	-	+
11	B3	65	+	-
12	B3	66	+	-
13	B3	17	+	-
14	C3	132	-	-
15	C3	114	+	+
16	C3	80	-	-
17	C3	78	-	-
18	C3	42	-	-
19	C3	37	-	-
20	C3	12	+	+

[a]CDC clinical classification (see Materials and Methods) and numbers of CD4 cells are shown in relation to presence (+) or absence (-) of HIV infection enhancement activity in IgA and IgG purified from the 20 HIV-infected individuals participating in the study.

infection enhancement capability. The majority (75%; 3/4) of category B symptomatic individuals (previously classified as ARC) also had IgA which enhanced HIV infection. However, IgA purified from sera of only 2/7 (28%) category C (AIDS) patients significantly enhanced infection of U937 by HIV.

Physiological concentrations of IgG block IgA-mediated enhancement

As shown in Table 3, with the exception of study subjects SP 9 and SP 20, who have serum IgA hypergammaglobulinemia, SP IgA samples found to enhance HIV infection did so at concentrations that would be present in sera with 8- to 100-fold greater concentrations of IgG. IgG from SP 5, 15, and 20 were previously observed to exhibit enhancement (see Table 2) at concentrations close to those shown in Table 3, which would be present in these individuals' sera with concentrations of IgA which also displayed enhancement. However, IgG from the remaining SP study subjects did not enhance HIV infection at or near these concentrations. Because higher concentrations of IgG which contain greater proportions of HIV antibodies than IgA (23) would be present in the majority of these sera, it is possible that IgG could bind epitopes recognized by IgA and block IgA-mediated enhancement. To determine whether this may be the case, IgA at concentrations found to significantly enhance HIV infection was combined with the relevant physiological concentration of IgG and re-examined for ability to enhance HIV infection. Control EI values used to determine whether enhancement of HIV infection by combined SP IgA and IgG was statistically significant were obtained by combining identical concentrations of the pooled SN IgA and IgG preparations.

As shown in Table 5, with the exception of SP11, enhancement by IgA was blocked in the presence of non-enhancing physiological concentrations of IgG. Only if IgA and IgG were combined at concentrations that could each mediate ADE of HIV infection was significant enhancement observed (see SP 5, 15, and 20). In the case of SP 11, it is possible that blockade of IgA-mediated enhancement was not observed because this individual's IgG may also contain enhancing antibodies. Although not considered

Table 5. Physiological concentrations of IgG block IgA-mediated enhancement[a]

SP	concentration IgA and IgG (μg/ml)	EI[b]		
		IgA alone	IgG alone	IgA and IgG
1	5.0 and 44	2.75 ± 0.15*	1.66 ± 0.22	1.40 ± 0.07
2	5.0 and 90	1.82 ± 0.10*	1.37 ± 0.18	1.40 ± 0.14
3	0.5 and 25	1.63 ± 0.06*	1.15 ± 0.09	0.93 ± 0.12
	0.05 and 2.5	1.25 ± 0.09*	1.35 ± 0.11	0.98 ± 0.17
4	5.0 and 37	1.73 ± 0.13*	1.71 ± 0.15	1.74 ± 0.15
5	5.0 and 70	1.83 ± 0.07*	1.54 ± 0.20	1.32 ± 0.17
	0.5 and 7.0	1.55 ± 0.11*	1.96 ± 0.12*	1.77 ± 0.05*
6	0.5 and 15	1.49 ± 0.14*	1.69 ± 0.11	1.28 ± 0.16
7	5.0 and 94	2.30 ± 0.18*	1.16 ± 0.14	1.30 ± 0.13
	0.5 and 9.4	1.90 ± 0.15*	1.24 ± 0.09	1.18 ± 0.13
8	5.0 and 76	1.96 ± 0.21*	1.40 ± 0.08	1.66 ± 0.03
9	5.0 and 8.1	2.28 ± 0.02*	1.40 ± 0.18	1.43 ± 0.10
11	5.0 and 70	2.11 ± 0.20*	1.77 ± 0.15	2.38 ± 0.13*
12	50 and 400	2.72 ± 0.08*	0.61 ± 0.07	1.73 ± 0.12
13	0.5 and 51	1.91 ± 0.13*	0.73 ± 0.02	0.90 ± 0.13
15	0.5 and 4.2	2.61 ± 0.06*	2.10 ± 0.21*	3.01 ± 0.04*
20	5.0 and 6.0	4.74 ± 0.10*	2.08 ± 0.17*	4.71 ± 0.15*
	0.5 and 0.6	1.50 ± 0.07*	0.98 ± 0.12	0.94 ± 0.06

[a]The indicated concentrations of IgA and IgG were combined and reacted with HIV for 1 h, after which U937 cells were added. Experiments were performed as detailed for the enhancement assay described in Materials and Methods. The EI ± SD shown represent results from one of two separate experiments.

[b]EI for SP IgA, SP IgG, or combinations of SP IgA and IgG were considered statistically significant (*) if 3 SD above the EI obtained with identical concentrations of pooled SN IgA and IgG. EI for pooled SN Ig preparations are not shown but were very similar to the mean EI values presented in Table 1.

statistically significant, the EI (1.77) for SP 11 IgG was 2 SD over that of controls. In the two cases (SP 12 and SP 13) where IgG alone exhibited ability to inhibit infection of cells by HIV, the addition of IgA masked both neutralizing activity in IgG and the enhancement which had been observed with IgA alone. This suggests that enhancing antibodies in sera may interfere with optimal neutralization of HIV, but the presence of neutralizing antibodies can also impede enhancement.

We have also performed enhancement assays in which those SP IgG found to enhance (shown in Table 2) were combined with physiologically relevant concentrations of IgA that would also be present in sera at these IgG concentrations. IgA had no effect on IgG-mediated enhancement (not shown).

Involvement of the FcαR in IgA-mediated enhancement

To determine if the FcαR participates in IgA-mediated enhancement of HIV infection, U937 cells were incubated with My43 or control MOPC-5.3μ mAb prior to addition of SP IgA and virus. No significant effects on viral production were observed in cultures of cells that had been preincubated with either of these mAb (Fig. 2).

As shown in Fig. 3, ADE by SP IgA could be significantly inhibited in the majority of cultures which contained cells that had been preincubated with My43. Preincubation with MOPC-5.3μ had little or no effect on HIV infection enhancement by SP IgA; EI values were similar to those previously obtained with these samples in the absence of this anti-dinitrophenyl mAb (Table 3). In addition, although an EI of 1.55 was obtained for MOPC-5.3μ-pretreated cells reacted with 50μg/ml pooled SN IgA and HIV, preincubation of cells with My43 reduced the EI for this IgA preparation to 1.08. Therefore, the ability of SN IgA to stimulate HIV production in U937 cells also appears to be associated with availability of the FcαR for binding.

Discussion

We have demonstrated that, in addition to IgG, IgA purified from sera of HIV-infected individuals can enhance HIV infection in U937 cells. Analogous to data

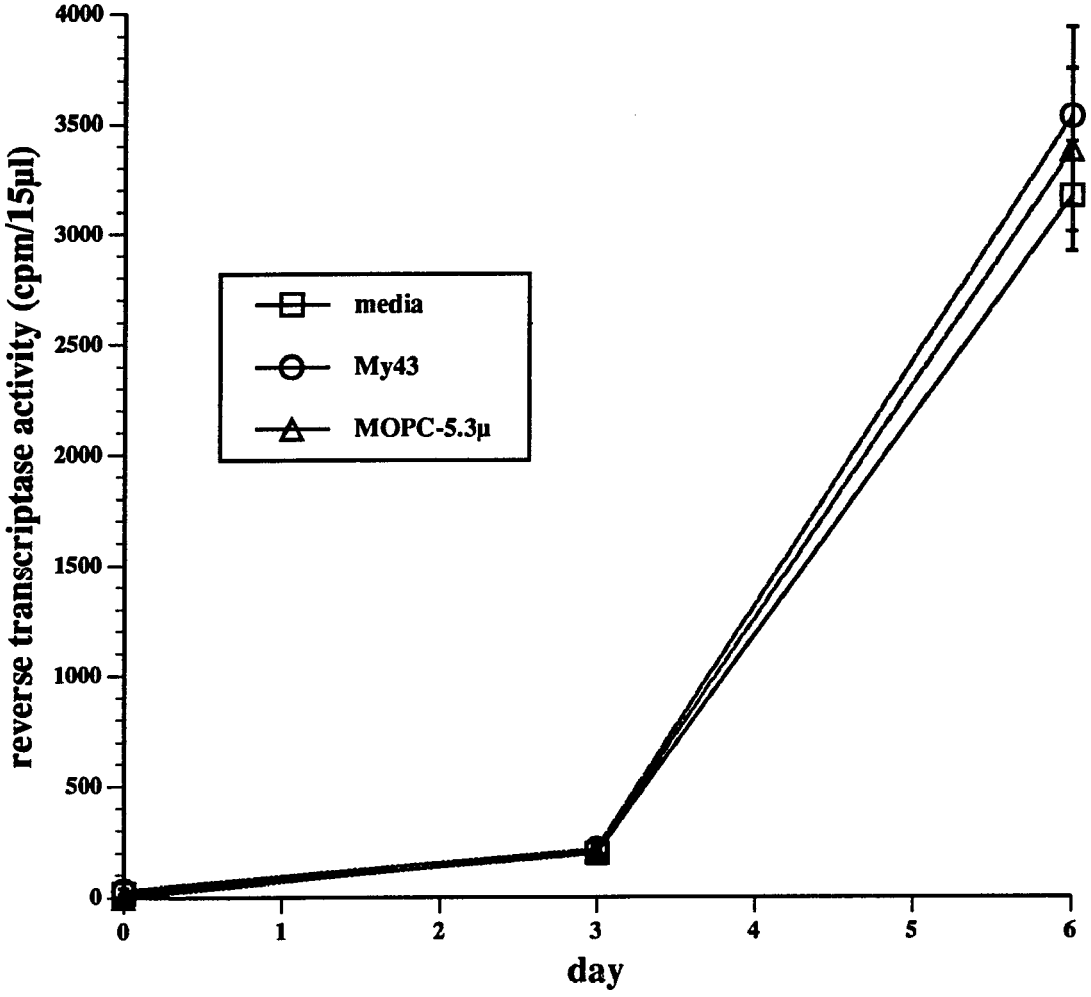


FIGURE 2. Kinetics of HIV replication in U937 cells. RT activity present in d3 and d6 medium of virus control cultures is shown for cells which had been incubated for 30 min on ice with My43, MOPC-5.3µ, or RPMI 1640 media aloneprior to addition of HIV. Experiments were performed as described for the enhancement assay in Materials and Methods. Results represent data from one of two separate experiments.

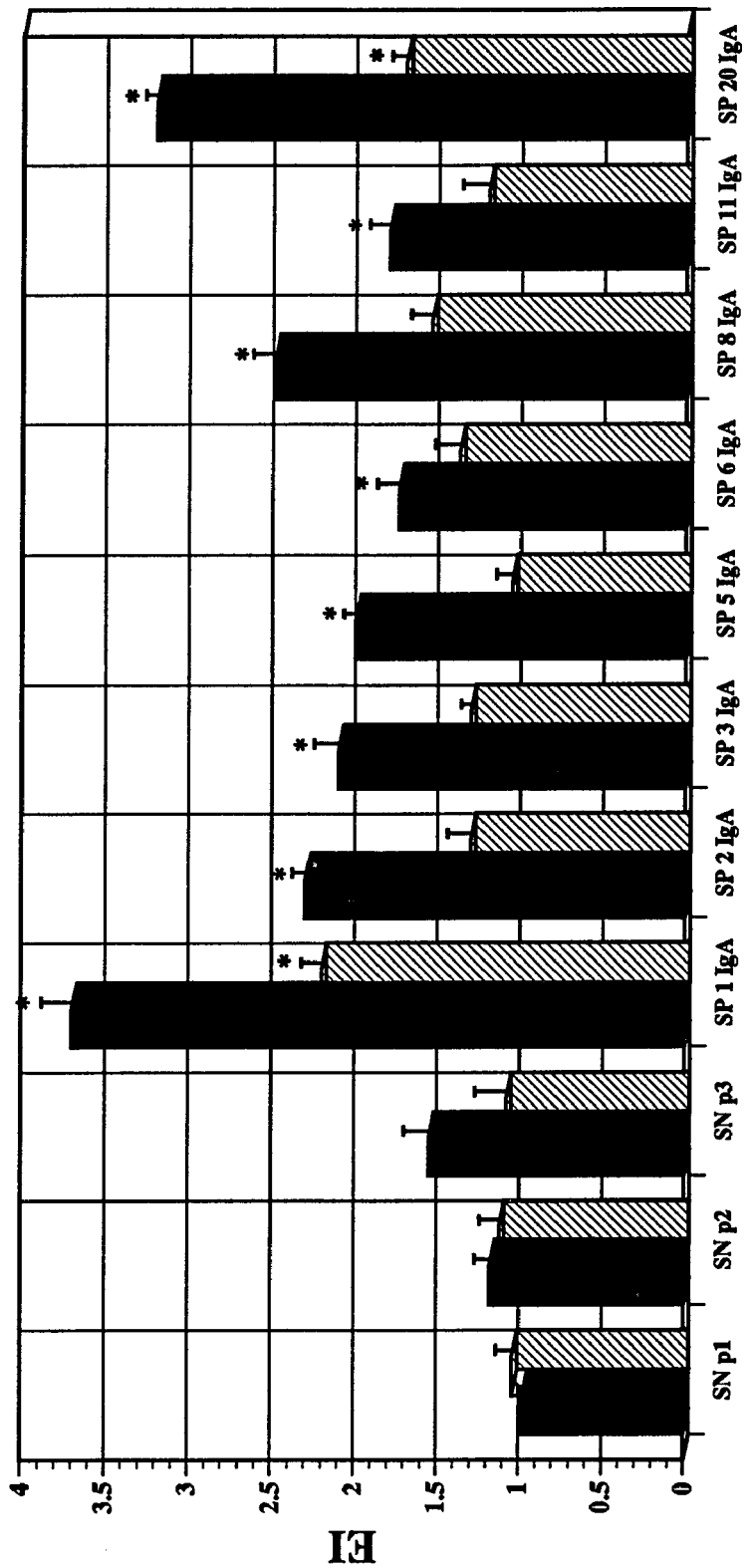


FIGURE 3. Inhibition of IgA-mediated ADE of HIV infection by anti-FcαR antibody. U937 cells were incubated 30 min on ice with MOPC-5.3μ (solid bars) or My43 (striped bars) mAb, then reacted with HIV in the presence of 0.5μg/ml (SN p1; SP 3 and 6) or 5μg/ml (SN p2; SP 1, 2, 5, 8, 11, and 20) SP or pooled SN IgA, as described in Materials and Methods. Results obtained with 50μg/ml (SN p3) of the SN IgA preparation are also shown. The EI was determined from RT activity present in medium of cultures on d6 and considered statistically significant for SP IgA if 3 SD above the EI for the same concentration of SN IgA and identical mAb. Results shown are representative of one of two separate experiments.

reported for IgG (5-8), the ability of IgA to participate in ADE of HIV infection is also dependent on the expression of isotype-specific FcR on cells. In contrast with IgG, however, twice as many (14/20) infected individuals had IgA exhibiting HIV infection enhancement activity. In addition, this activity was predominantly observed with IgA from HIV-antibody positive asymptomatic study subjects.

Our results are in agreement with those of others who have demonstrated that although high concentrations of IgG inhibit HIV-1_{IIIB} infection of U937, enhanced infection can occur at low IgG concentrations, often equivalent to sera dilutions of 10^{-4} to 10^{-6} (5,7). Whole sera of HIV-antibody positive individuals have also been shown to enhance HIV infection at dilutions in this range (10). The finding here that, as compared with IgG, larger concentrations of IgA are required to demonstrate enhancement of HIV infection is not unexpected. We have previously quantitated these sera for both total IgG and IgA concentrations and proportions of HIV antibodies present within each isotype. Results indicated that HIV-infected individuals generally have 28- to 43-fold greater concentrations of circulating IgG HIV antibodies compared to IgA (23). Therefore, it is not surprising that IgA was most often observed to enhance viral infection using concentrations equivalent to the lower serum dilutions 10^{-3} and 10^{-4}.

The frequency of enhancement activity among sera of HIV-infected individuals has been difficult to establish due to the small study groups utilized in previous studies of ADE. In the present study, the frequency (7/20 or 35%) of antibody-positive sera found to contain IgG exhibiting enhancement capability is very similar to that (5/16 or 31%) previously reported by others who similarly used purified IgG, U937 cells, and the HIV-1_{IIIB} isolate (7). However, the prevalence of ADE phenomena within the HIV-infected population may be impossible to ascertain definitively since this activity probably varies among sera in regard to many factors such as the epitope specificity of HIV antibodies, the HIV isolates used for study *in vitro*, and the receptors on target cells which are utilized in ADE. It is clear that the ability to demonstrate ADE of HIV infection is not

simply related to the use of U937 cells and the HIV-1_{IIIB} isolate; enhanced infection of peripheral blood monocytes and macrophages by HIV-1_{SF128A}, HIV-1_{Ba-L}, and homotypic isolates has been reported (5,8,9,12). In addition, it is important to note that despite its tropism for T cells, HIV-1_{IIIB} has also been used to demonstrate IgG-mediated ADE of HIV infection in primary macrophage cultures (5,9). Therefore, it is possible that ADE phenomena *in vivo* could further the dissemination of virus among macrophages through expanding the cell tropism of HIV. It is quite likely that IgA can also enhance HIV infection of these cells since monocytes and macrophages both phagocytose IgA immune complexes (reviewed in Ref. 30), a function dependent on the FcαR (28). Additional studies designed to examine the ability of IgA to enhance HIV infection in primary cultures of cells of this lineage are in progress.

The ability of sera or purified Ig from infected individuals to enhance infection of cells by diverse HIV isolates could be interpreted to suggest that antibodies recognizing a conserved HIV epitope are responsible for mediating enhancement. Evidence that infection-enhancing epitopes reside within gp41 has been provided in studies of both ADE and C-ADE using several human mAb directed against conserved regions of this glycoprotein (21,22). In this regard, it should be noted that IgA HIV antibodies predominantly recognize the envelope glycoproteins and that reactivity against gp41 is most frequently observed (18). Therefore, it is possible that the greater incidence of sera having IgA exhibiting infection enhancement activity may be due to the presence of higher proportions of IgA anti-HIV antibodies than IgG antibodies which react specifically with an "enhancing" determinant of this virus.

The higher prevalence of IgA exhibiting HIV infection enhancement activity could also be related to the presence of lower concentrations of neutralizing antibodies. We have previously found that although serum IgA of infected individuals can inhibit HIV-1_{IIIB} infection of CEM T cells, identical concentrations of IgG from these individuals are much more effective in neutralizing infection. Neutralization of this

isolate by both IgA and IgG is not achieved through reactivity with the V3 loop (23). It appears that, relative to IgG, IgA contains lower proportions of group-specific (cross-reactive) neutralizing antibodies, possibly those directed against the gp120 CD4-binding site. Proportions of IgA antibodies directed against a V3 peptide of North American consensus sequence are also lower than those in IgG (23). Therefore, if the presence of low concentrations of neutralizing antibodies is related to the ability to enhance HIV infection, the frequency of infection enhancing activity for homotypic isolates *in vivo* might also be greater within IgA than IgG.

At present the intracellular mechanisms involved in ADE of HIV infection are unclear. Extracellular events clearly involve binding of complexed HIV to FcR (5-8) or, in the case of C-ADE, the CR2 complement receptor (14,16). It has been proposed that ADE is initiated with internalization, via receptor-mediated endocytosis, of FcR-bound antibody-HIV complexes (33). Following endocytosis, it has been speculated that the envelope of the HIV virion fuses with the membrane of an endosomal vesicle such that the viral core is inserted into the cytoplasm of the cell before the virion is delivered to a lysosome, where degradation would occur (33). This is a reasonable hypothesis since, as opposed to direct fusion with the plasma membrane, HIV can also enter cells via receptor-mediated endocytosis in the absence of antibody (34,35). Further, this latter entry route for HIV has been shown in electron micrographs to involve fusion of the internalized virion with an endosomal membrane. The majority of evidence indicates that CD4 is required in demonstrating both ADE and C-ADE of HIV infection. Since, in the case of C-ADE, binding of antibody-complexed HIV to the CR2 complement receptor on MT-2 T cells can occur in the presence of OKT4a (36), anti-CD4 mAb and soluble CD4 may block ADE intracellularly through inhibiting the binding of gp120 to CD4 in the membrane of the endosome. In accordance with this model, internalized HIV virions complexed with antibodies which inhibit fusion (those against the gp120 CD4-binding site or V3 loop) could not escape being routed to lysosomes and degraded.

It is possible that more IgA than IgG may exhibit the capacity for enhancing HIV infection because signals transduced following the binding of antibody-complexed HIV to the FcαR differ from those of FcγR. We have shown that uncomplexed IgA and IgG from sera of SN individuals bind U937 cells. The finding that consecutive exposure of these cells to high concentrations of these preparations and virus augmented levels of viral production implies that signals transduced on binding to FcR may influence HIV infection. With the exception of stimulating phagocytosis and production of inflammatory and antimicrobial factors associated with the oxidative burst (30), the consequences of triggering FcαR are largely unknown. TNF-α has been reported to be produced by macrophages following cross-linking of FcγR (37). The possibility cannot be excluded that synthesis of this cytokine, which up-regulates HIV replication (38), or other HIV-promoting cell proteins occurs subsequent to cross-linking of both FcαR and FcγR on U937 and may be involved in ADE of HIV infection.

The higher incidence of HIV infection-enhancing activity observed in IgA from sera of SP asymptomatic individuals than from those with AIDS may reflect the presence of more polymeric IgA1 in IgA samples from the former. In humans, serum IgA exists in different molecular forms which are equally distributed among two subclasses, IgA1 and IgA2, both of which are distributed as approximately 90% monomer (m) and 10% polymer (p). We have previously found that the percentage of pIgA2 in sera of HIV-infected individuals is significantly reduced as early after infection as the asymptomatic stage. In contrast, the percentage of pIgA1 in these sera is relatively intact throughout disease progression until end-stage disease, where a dramatic reduction is observed (18). This may be a significant finding in relation to IgA-mediated ADE since IgA HIV antibodies are restricted to the IgA1 subclass (18) and pIgA is more effective than mIgA in functioning as an opsonin. In addition, studies examining the binding of m- and pIgA to the FcαR on U937 have demonstrated that although this receptor binds mIgA, it appears to have a greater affinity for pIgA (39,40). IgA purified from sera of SP

asymptomatic individuals is also more effective than IgA from AIDS patients at inhibiting infection of T cells by HIV (23). Therefore, it is possible that both neutralizing and enhancing activity in serum IgA may be functions primarily associated with pIgA1 HIV antibodies.

As we have shown, IgA-mediated ADE of HIV-1_{IIIB} infection in U937 can be blocked in the presence of physiological concentrations of IgG which do not exhibit similar enhancing activity. If the incidence of IgG exhibiting infection enhancement of homotypic isolates is also less frequent than that for IgA, IgA-mediated ADE could be similarly masked *in vivo*. However, IgA concentrations in sera are below those of IgG as a result of the shorter half-life of IgA; roughly equivalent amounts of IgA and IgG are actually synthesized on a daily basis by plasma cells in the bone marrow (reviewd in Ref. 41). Therefore, it cannot be concluded with certainty that IgA with enhancing activity could not facilitate systemic proliferation of virus within FcαR$^+$ monocytes and macrophages. In addition, other IgA receptors such as the IgA1-binding asialoglycoprotein receptor expressed on liver hepatocytes (42) could also potentially participate in enhancing entry of antibody-complexed HIV into cells.

The possibility that IgA antibodies recognizing an HIV-enhancing determinant could profoundly influence infection of cells associated with the mucosa should also be considered. IgA is the Ig isotype predominantly responsible for protecting mucosal surfaces against invading pathogens (25). The majority of plasma cells located in lamina propria or interstitial regions of mucosal tissues synthesize pIgA. Thus, concentrations of IgA in secretions are generally greater than those of other isotypes. Enhanced infection of FcαR$^+$ mucosal macrophages could contribute to expansion of HIV in these areas. HIV-specific pIgA antibodies could also potentially facilitate viral entry and infection of epithelial cells in the mucosal epithelium following the binding of IgA-HIV complexes to the pIg receptor (pIgR) expressed on the basolateral surfaces of these cells. Such a

phenomenon has been demonstrated *in vitro* with the pIgR$^+$ HT-29 colonic epithelial cell line and pIgA complexed with EBV (43).

In addition to the possibility that dissemination of virus may be accelerated by HIV IgA antibodies in mucosal regions, the potential hazards which might arise following immunization of vaccine recipients should also be considered. Induction of IgA antibodies with infection enhancement capability in the male or female genital tract could pose a threat to the development of an effective vaccine which protects individuals from acquiring disease following sexual transmission of HIV. The problems recently encountered in vaccine trials (2,44) suggest that ADE phenomenon may warrant further study in the effort to achieve successful immunization of individuals against HIV.

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DISCUSSION

CHARACTERISTICS OF IgA IN HIV-INFECTED INDIVIDUALS

Serum IgA1 and IgA2 Subclasses. IgA and IgG hypergammaglobulinemia are conditions commonly associated with HIV infection, speculated to arise as a consequence of an intense nonspecific polyclonal activation of B cells. Elevations in serum IgA have been reported to be due to increases within the IgA1 subclass (Reimer et al., 1988). However, when we examined the subclass distributions of IgA in the serum of HIV-seropositive individuals, we observed that, in contrast to this early study, most of our patient population exhibited proportional elevations of both IgA1 and IgA2; this finding has since been confirmed by others (Cassulis et al., 1991). Our results do support the finding that individuals with end-stage disease exhibit most pronounced elevations of IgA (Fling et al., 1988; Reimer et al., 1988; Reid et al., 1988). Our results also agree with observations that the development of less dramatic (twofold) elevations in serum IgG concentration occur in the majority of individuals early after HIV infection and remain consistently elevated as disease progresses (Fling et al., 1988; Reimer et al., 1988). Our data do not agree, however, with reports that serum IgA concentrations are generally normal during early disease stages (Fling et al., 1988; Reimer et al., 1988); mean IgA concentrations of individuals classified as either asymptomatic (ASY) or having lymphadenopathy (LAD) were found to be above normal in our first study (Kozlowski & Jackson, 1992). Our second study population also contained many asymptomatic individuals (27%) exhibiting elevated IgA (Kozlowski et al., 1994).

Polyclonality of serum IgA. Despite the presence of elevated serum IgA2 concentrations in infected study subjects, we have found by western blotting that reactivity of IgA against HIV is restricted to the IgA1 subclass. Elevated CIC containing

IgA1 but not IgA2 in infected individuals (Jackson et al., 1988a) may thus be formed by IgA1 anti-HIV antibodies in addition to IgA1-directed RF (Jackson et al., 1988b). These results also initially suggested that polyclonal B cell activation accompanying HIV infection is manifested among those B cells committted to IgA2 production. After quantitation of IgA HIV antibodies, we found that polyclonal activation of IgA1-committed B cells also occurs in HIV-infected individuals.

Our studies indicate that both antigen-specific and polyclonal activation of B cells accompany HIV infection. We have demonstrated that elevations in serum IgG concentrations are due specifically to increases in IgG HIV antibodies, supporting the proposal that an intense antigen-driven response by B cells occurs in infected individuals (Amadori et al., 1989). However, a generalized nonspecific activation of IgA-committed B cells also accompanies infection and is responsible for elevations of serum IgA by production of non-HIV antibodies of unknown specificity.

Monomeric and polymeric IgA. Elevations in nonviral-specific IgA1 and IgA2 appear to be associated specifically with monomeric IgA; the percentage of polymeric forms in sera was reduced within both subclasses, although most pronounced among the IgA2 subclass. The manifestation of elevated serum IgA as monomer in subclass ratios correlating with those typically found in sera (Skvaril & Morell, 1974) suggests that the source of these elevations is likely to be polyclonally activated plasma cells in the bone marrow, rather than in the mucosa, which under normal conditions predominantly synthesize polymeric IgA and roughly equal concentrations of IgA1 and IgA2 (McGhee & Mestecky, 1992).

One possible explanation for the reduction in polymeric IgA in sera of HIV-infected individuals may be related to the chronic nature of this disease. Although the majority of serum IgA is monomeric, recent studies have shown that antigen-specific serum IgA antibodies induced immediately following viral or bacterial infections or systemic immunization with various antigens often first appear as polymers (Brown et al.,

1985b; Tarkowski et al., 1990). IgA HIV antibodies produced immediately following the acute phase of HIV infection or during seroconversion may similarly be manifested in serum as polymers. We did not have the opportunity to examine serum IgA in seroconverting individuals due to the difficulties involved in identifying such subjects. Our patient population is more likely analogous to individuals experiencing a long-term immune response in which antigen-specific monomeric IgA predominates in serum. It may be possible in the future to obtain enough blood samples to specifically address this issue since detection of acutely infected individuals has now been facilitated by the finding that such individuals characteristically experience a flu- or mononucleosis-like illness shortly after acquiring HIV (Clark et al., 1991).

Relationship Between Elevated IgA and T Helper Cells. Of all the Ig isotypes, the production of IgA is believed to be highly T-cell dependent. Previous studies with thymectomized rabbits or athymic nude mice have demonstrated that concentrations of both antigen-specific IgA antibodies and total IgA in plasma of these animals are dramatically reduced (reviewed by McGhee et al., 1989). Evidence that the $CD4^+$ T helper cell subset is required for differentiation of surface-positive IgA B cells into IgA plasma cells in mice has also been provided (Mega et al., 1991). Thus, the finding by others (Fling et al., 1988; Reid et al., 1988; Reimer et al., 1988) that development of elevated IgA in HIV infection is related to declining numbers of $CD4^+$ T cells seems peculiar. Indeed, our results do not support this conclusion. However, for reasons discussed below, the findings of these investigators with respect to negative correlation between IgA levels and CD4 count may be explained.

We measured IgA concentrations in blood of 80 seropositive study subjects who were categorized into one of four HIV clinical disease stages, ranging from CDC stage II (ASY) to stage IV (AIDS). As discussed above, many individuals in the early disease stage groups ASY and LAD (stage III) exhibit elevated IgA. When CD4 count of ASY subjects alone was statistically examined in regard to serum IgA concentration, no

correlation between these variables was found. Similarly, no significant association of IgA concentration with CD4 count could be demonstrated within any of the other groups comprising this study population. Combining data from the ASY, LAD, and ARC groups and repeating this statistical analysis also failed to indicate any relationship between IgA and CD4 counts. However, when data of the AIDS group were included, a significant correlation between elevated IgA and low CD4 count could then be shown. The ability to demonstrate this inverse relationship was made possible because the profoundly lower CD4 counts of individuals in the AIDS group skewed this analysis. Therefore, the finding by others that serum IgA concentrations are related to absolute numbers of $CD4^+$ T cells of HIV-infected individuals may not be valid.

We have also found that proportions of HIV antibodies in samples of purified IgA do not correlate with CD4 counts of study subjects but instead inversely correlate with total serum IgA concentrations. As with IgA, total IgG concentrations of this second study population were not significantly associated with CD4 counts. However, numbers of $CD4^+$ T cells in these individuals did indeed positively correlate with the proportion of HIV antibodies in purified IgG. Reductions in levels of serum HIV antibodies have similarly been correlated with disease progression by others (Radkowski et al., 1991).

Increased spontaneous production of IgG and IgM by PBMC has been shown to be significantly associated with decreased percentages of $CD4^+$ and increased percentages of $CD8^+$ T cells but not with absolute numbers of these cells in HIV-infected individuals (Mizuma et al., 1988). This has led to the speculation that it may be the maintenance of a normal proportion of the $CD4^+$ and $CD8^+$ T cell subsets that could be most critical in regulating B cell activity in infected subjects (Mizuma et al., 1988). The relationship between IgA levels and proportions of $CD4^+$ and $CD8^+$ T cells was not directly examined in our studies. Therefore, the possibility that increases in serum IgA concentration may be related to alterations in the proportion of cells constituting these subsets cannot be excluded, particularly in view of the finding that increasing serum IgA

and decreasing CD4/CD8 ratios have both been significantly correlated with disease progression (Reid et al., 1988).

IgA and T helper cell subsets. Our data indicate that elevations in IgG are due to increases in IgG HIV antibodies, while elevations in serum IgA are a result of increases in non-HIV IgA antibodies. This suggests that HIV infection differentially affects IgA and IgG B cell populations. What factors might render IgA- but not IgG- committed B cells susceptible to polyclonal activation? Certainly, the generation and maintenance of normal IgA and IgG responses in humans may be regulated differentially in regard to certain factors such as lymphokines, as they are in mice (McGhee et al., 1989). For instance, IL-4 has been shown to enhance synthesis of IgG1 and IgE in LPS-stimulated murine B cell cultures; IL-5 and IL-6, on the other hand, augment IgA production (Paul, 1987; Beagley et al., 1988, 1989). Therefore, in addition to imbalances arising within the $CD4^+$ and $CD8^+$ T cell subsets, alterations in normal lymphokine profiles produced by cells constituting the T helper subsets T_H1 and T_H2 (Mosmann et al., 1986; Romagnani, 1991) may also lead to abnormalities which differentially affect IgA and IgG B cell populations in HIV-infected individuals.

There is accumulating evidence that a selection for either the T_H1 or the T_H2 subset accompanies certain immune responses in humans and may be in part determined by antigenic character (Romagnani, 1991). A similar phenomenon has been proposed to occur in HIV infection, with T_H2 cells gradually emerging over the course of disease to become the dominant subset (Clerici & Shearer, 1993). Antigen- or mitogen-stimulated PBMC from symptomatic HIV-infected individuals have been found to produce reduced amounts of the T_H1-associated cytokines IL-2 and IFN-γ but increased amounts of IL-4 and IL-10, produced by T_H2 cells (Clerici & Shearer, 1993). Evidence that production of monocyte-derived IL-12 is decreased in HIV-infected individuals has also been presented (Chehimi et al., 1994). This latter study lends support to the theory that the T_H2 cell subset eventually predominates in HIV-infected subjects since IL-12 has been found to

inhibit T_H2 responses while inducing those characteristic of T_H1 (Scott, 1993). IL-10 produced by T_H2 cells could contribute to the maintenance of this subset since this cytokine downregulates T_H1-type responses (Fiorentino et al., 1991). In addition, IL-4 produced by T_H2 cells could play a role both in elevating IgG1 concentrations (Reimer et al., 1988) through augmenting IgG HIV antibody responses and in the development of elevated IgE in sera of seropositive individuals having CD4 counts below 200 (Lucey et al., 1990).

The T_H2-type cytokines IL-4 and IL-5 have been found to enhance proliferation and differentiation of IgA-committed B cells following activation (McGhee et al., 1989; Beagley et al., 1988). In addition, synthesis of IgA by activated human IgA B cells is enhanced in the presence of the T_H2-associated cytokine IL-6 (Beagley et al., 1989). Therefore, it is possible that, in the absence of T_H2 downregulatory mechanisms, activation of IgA B cells by non-HIV antigens in the presence of these T_H2-associated cytokines could lead to enhanced proliferation and expansion of IgA B cell clones with specificity for other pathogens. In this way, a switch from the T_H1 to T_H2 cytokine phenotype could contribute to the polyclonal activation of IgA B cells in HIV-infected individuals.

It should be cautioned that others (Maggi et al., 1994; Graziosi et al., 1994) have been unable to confirm that such a shift to a predominant T_H2 cell phenotype accompanies HIV infection. However, this issue still remains unresolved, since data presented by these latter investigators also conflict in regard to cytokine profiles characteristic of T cells in HIV-infected individuals. It is possible that predominant production of T_H2 cytokines supporting IgA responses could contribute to polyclonal activation of IgA B cells in HIV-infected individuals, but it is highly implausible that $CD4^+$ T_H cells alone are involved in this phenomenon. AIDS patients with virtually no $CD4^+$ T cells often exhibit dramatic elevations in serum IgA. This suggests that, as found in mice (Mega et al., 1991), commitment of human B cells to the IgA isotype can occur

in the absence of $CD4^+$ T helper cells. It is quite likely that aberrant production of IL-6 by monocytes or dendritic cells may enhance terminal differentiation and Ig synthesis of IgA-committed B cells in HIV-infected individuals, particularly since spontaneous secretion of Ig by PBMC from infected subjects can be abrogated with monocyte-depletion or inclusion of anti-IL-6 antibody in such cultures (Amadori et al., 1991). The possibility that HIV-infected monocytes producing IL-6 augment synthesis of IgA could be tested using primary macrophage cultures infected with HIV and antigen- or mitogen-stimulated autologous PBMC. The use of anti-cytokine reagents could also, as above, provide valuable information in culture systems designed specifically to examine the effects of infected macrophages or other cells on IgA production.

Polyclonal Activation and gp120. Another potential mechanism through which HIV may polyclonally stimulate predominantly IgA B cells has been recently described. This phenomenon may warrant some concern as it appears to be mediated specifically by gp120, a potential immunogen for vaccination. Recombinant gp120 of HIV-1_{SF-2} has been found to bind normal tonsillar B cells via membrane V_H3 Ig (Berberian et al., 1993). This viral antigen may be responsible for causing the previously observed increases in proliferation and Ig secretion by normal B cells exposed to HIV (Schnittman et al., 1986). Addition of gp120 to cultures of purified normal tonsillar V_H3^+ B cells was found to lead to dramatic increases in production of total Ig. IgM purified from normal sera was also shown to bind gp120, and this binding was 10-fold greater than that observed with IgG.

Distribution of the V_H3 subgroup among myeloma proteins has been found by sequence analysis to be 18%, 30%, and 67% for IgG, IgM, and IgA, respectively, correlating with distributions (20% for IgG and 75% for IgA) observed in normal sera (Capra & Kehoe, 1975). Others (Førre et al., 1976) have found the V_H3 subgroup distribution to be greater (45%) among IgG myeloma proteins with serological typing, but a similar percentage of myeloma IgA proteins (70%) was observed to express V_H3. The predominant expression of V_H3 by IgA suggests that activation resulting from the

binding of gp120 to membrane V_H3 Ig on B cells could more profoundly influence IgA responses.

Expansion of the V_H3 B cell pool has been observed early in HIV infection, although a clonal deficit of these cells (with enrichment of V_H1 B cells) in the circulation, spleen, and lymph node is evidenced at later clinical stages (Berberian et al., 1991). Because T cell superantigens are characteristically associated with initial stimulation followed by subsequent deletion, these findings, in conjunction with the observed gp120-mediated activation of V_H3 B cells, have been interpreted to suggest that gp120 may act as a B cell superantigen. Unfortunately, because a relationship between gp120 and IgA was not investigated in this study, it is not known whether this phenomenon could be related to the elevations in IgA associated with HIV infection. Although others have reported numbers of total B cells in HIV infection are reduced (Reddy et al., 1991), a superantigenic property of gp120 which leads to deletion of IgA B cells could be debated with the argument that most extreme elevations in IgA are observed in AIDS patients where deficits of V_H3 B cells should be most pronounced.

The V_H3 binding region of gp120 is likely peptide encoded since both glycosylated and nonglycosylated recombinant $gp120_{SF-2}$ bind V_H3 Ig. In addition, this region appears to be conserved among HIV isolates; gp120 of HIV-1_{JR-CSF}, HIV-1_{JR-FL}, HIV-1_{BH10}, and the monocyte-tropic HIV-1_{Ba-L} were all found to be capable of binding V_H3 Ig. One potential candidate for this region could be the highly conserved amino acid sequence at residues 487-511 in the gp120 carboxy terminus. Proliferation of normal B cells has been previously demonstrated using a peptide of this sequence (Nair et al., 1988). In view of the potential for this phenomenon to interfere with successful vaccination against HIV, the region of gp120 which binds V_H3 Ig on B cells is currently under investigation and hopefully will soon be elucidated.

Our data have suggested that binding between an HIV antigen and non-HIV IgA but not IgG antibodies may indeed occur. Although it is not clear whether this interaction

may involve gp120 and V_H3-expressing IgA, we did observe that reactivity against whole HIV lysates by IgA purified from sera of HIV-seronegative individuals was sixfold above that of IgG from these subjects. We did not observe such a difference between these Ig preparations in binding to gp120 V3 peptides. Further analyses will be necessary to determine whether IgA antibodies nonspecifically interact with a component of HIV antigen and, if so, whether it may be gp120.

It is quite likely that IgA B cell polyclonal activation is mediated through multifactored influences. For instance, membrane TNF-α on HIV-infected T cells (Macchia et al., 1993) and IL-6 produced by macrophages (Nakajima et al., 1989), in conjunction with binding of gp120 to membrane V_H3 Ig of B cells, could together contribute profoundly to polyclonal B cell activation associated with HIV infection. Elevated levels of these cytokines detected in PBMC or sera of infected individuals (Breen et al., 1990; Wright et al., 1988; Roux-Lombard et al., 1990) may be directly related to intensifying the state of B cell activation, both general and specific.

Catabolism of IgA. It is not clear why serum IgA or IgA CIC are not effectively catabolized in HIV-infected subjects. Excessive generation of immune complexes has been postulated to lead to blockade of the phagocytic compartment of the spleen and liver that functions to remove antibody-complexed antigens from the circulation (Morrow et al., 1986). There is evidence that impairment of Fc receptor-mediated clearance of IgG-antigen complexes by the RES occurs in patients with ARC and is more severe in those with AIDS (Bender et al., 1985). Both IgA immune complexes and IgA are believed to be removed from the circulation primarily by the liver (Brandtzaeg et al., 1994; Moldoveanu et al., 1990). Although not directly examined, normal clearance of IgA CIC and serum IgA may be impaired as a result of active infections in the liver of infected individuals.

Hepatic disease is evidenced in a high proportion of AIDS patients (Glasgow et al., 1985). Although liver diseases associated with HIV infection are most often

manifested as secondary infections with *Mycobacterium avium, Cryptococcus neoformans,* CMV, hepatitis B, or hepatitis C (Cao et al., 1992), loss of normal liver function could also arise through direct HIV infection of resident cells. Productive infection of hepatoma cell lines as well as primary cultures of Kupffer cells by HIV has been successfully accomplished *in vitro* (Schmitt et al., 1990; Cao et al., 1990). The presence of HIV-infected hepatic cells *in vivo* has been supported by the demonstration of HIV DNA in liver biopsy specimens from infected individuals (Cao et al., 1992). Further, HIV mRNA and p24 antigen have been localized to both hepatocytes and Kupffer cells in these tissues (Cao et al., 1992).

Because liver macrophages function in humans to remove particulate material from the circulation, active infections by HIV and/or secondary pathogens may alter the capacity of such cells to clear immune complexes from serum. In addition, abnormal function arising subsequent to infection of hepatocytes may have severe consequences specifically in regard to the clearance of IgA and IgA immune complexes, since several receptors expressed on hepatocytes have been implicated in the removal of IgA from plasma (Brandtzaeg et al., 1994; Tomana et al., 1988).

Although not definitively established as yet in humans, several studies suggest that the asialoglycoprotein receptor (ASGP-R) expressed on hepatocytes may function to remove IgA1 from plasma (Tomana et al., 1988). Binding of IgA to this receptor is mediated through interactions with terminal galactose residues associated with O-linked carbohydrate side chains present in the hinge region of IgA1, a region absent from the IgA2 molecule (Underdown & Mestecky, 1994). The presence of a m- and pIgA-binding FcαR on Kupffer cells is also believed to function in the catabolism of both plasma IgA and IgA CIC (Sancho et al., 1986). Evidence that both Fcγ and complement receptors expressed on these cells may further contribute to phagocytosis of IgA CIC which contain IgG has been provided (Roccatello et al., 1992). Since Kupffer cells are susceptible to HIV infection, phagocytic capability or normal regulation of these receptors may be

altered, thus causing elevations of IgA CIC. Clearance of IgA CIC by liver cells has been found to be saturable, however, in mice (Russell et al., 1981). Therefore, elevated CIC and IgA detected in HIV-infected individuals may simply arise following excessive antibody production by polyclonally activated IgA B cells and saturation of IgA receptors on both macrophages and hepatocytes. Experiments designed to examine the binding of IgA to hepatocytes and Kupffer cells infected with HIV could help to elucidate whether viral infection may alter the capacity of these cells to function in the catabolism of IgA.

Mucosal IgA B Cells and S-IgA in HIV Infection. Whether polyclonal activation of IgA B cells also occurs at mucosal sites is currently unknown. The manifestation of increased numbers of circulating B cells spontaneously secreting Ig in HIV-infected individuals has been found to be related to the presence of HIV-specific IgG plasma cells (Amadori et al., 1988). Reduced responses by PBMC to mitogens probably reflect this increase in numbers of antigen-specific activated B cells. However, different patterns of reactivity against HIV have been observed between IgG antibodies in serum and those secreted by PBMC of some individuals (Amadori et al., 1988). As we have demonstrated, the broad serum reactivity against all HIV antigens reflects that seen with IgG. The restricted antibody response to HIV envelope glycoproteins observed by spontaneously secreted IgG in PBMC cultures is analogous to that of IgA, both in serum and secretions. Therefore, the question arises as to whether the anti-IgG detecting reagent used in this early study may have cross-reacted with IgA. If so, this could indicate that a significant component of activated HIV-specific circulating B cells of some infected individuals may be IgA committed and mucosally derived. Further studies designed to specifically address this issue may help resolve the question of whether polyclonal or antigen-specific activation is associated with B cells of the mucosa.

Elevated levels of IgA, IgG, and IgM have been detected in cervicovaginal washes of HIV-infected women (Lu et al., 1993). IgG and IgA were suggested to be derived by passive transudation from blood since total IgG concentrations significantly

correlated with concentrations of IgG in sera (Lu et al., 1993). Under normal conditions, protection of the female reproductive tract is believed to be mediated by both locally produced and passively derived Ig (Parr & Parr, 1994). Evidence that exudation of monomeric IgA1 from plasma into intestinal fluids does occur in HIV infection has also been presented (Janoff et al., 1994). However, if passive transudation of plasma Ig is responsible for elevating IgA and IgG in cervicovaginal washes of these HIV-infected women, it is curious that, unlike with IgG, total serum IgA levels did not correlate with IgA concentrations in these fluids. Further, no correlation could be demonstrated between levels of anti-gp160 IgG (or IgA) antibodies in sera and cervicovaginal washes. In view of its size, it is also questionable whether elevated IgM in these fluids is transudated from plasma. Therefore, these findings could reflect the presence of polyclonally activated B cells in the female reproductive/genital tract.

Salivary secretions of AIDS patients but not of asymptomatic individuals have been shown to exhibit decreased concentrations of IgA, especially of the IgA2 subclass (Jackson, 1990; Müller et al., 1991). This is likely related to decreased parotid and submandibular/sublingual salivary fluid output, rates which are significantly decreased in individuals with AIDS (Müller et al., 1991; Coogan et al., 1994; Atkinson et al., 1990). Decreased parotid output of IgA in these patients has been linked to the presence of oral infections (Müller et al., 1991). In addition, abnormal increases in concentrations of antimicrobial proteins such as histatin and lysozyme have been found to be associated with secretions from some salivary glands but not others (Atkinson et al., 1990). Thus, an accurate assessment of B cell activity associated with the oral cavity may be difficult.

It is interesting that the predominant B cell aberrancy observed in the oral mucosa appears to involve production of (presumably polymeric) IgA2 (Müller et al., 1991; Jackson, 1990). We also found that the decreased percentage of polymeric IgA in sera is most pronounced in this subclass. Reduced proportions of duodenal and colonic IgA plasma cells in HIV-infected subjects have also been attributed specifically to decreased

numbers of cells secreting IgA2 (Janoff et al., 1994). It is difficult to reconcile these data with a proposed polyclonal activation by gp120 since the utilization of the V_H3 gene has not been found to differ between the IgA subclasses. Possibly, within the microenvironments of the bone marrow and the intestinal mucosa, cells which contribute to commitment, activation, or differentiation of IgA2 B cells could be differentially affected by HIV, accounting for the polyclonal activation of systemic IgA2 B cells at the same time that numbers of such cells in the intestinal mucosa are decreased. At this time, regulation of the IgA subclasses is poorly understood. IgA1 and IgA2 responses are not developed identically in mucosal tissues and the bone marrow as evidenced by the differences in distributions of these subclasses in sera and secretions. IgA2 mucosal B cells in HIV-infected individuals may somehow be prevented from undergoing later stages of maturation or be unable to home to mucosal sites for terminal differentiation into plasma cells. Whatever the mechanism, reductions in IgA2 plasma cells could greatly contribute to the pathogenesis of HIV infection by rendering mucosal surfaces of individuals more susceptible to invasion by opportunistic pathogens, particularly those expressing polysaccharide antigens. If HIV antigens such as gp120 are directly or indirectly responsible for causing IgA2-related B cell abnormalities, these antigens should be identified so that mucosal immunization against HIV does not leave vaccine recipients susceptible to infection by other microorganisms.

IgA Subclass Response to HIV. Our data have demonstrated that HIV IgA antibodies in blood are restricted to the IgA1 subclass. This finding has not been entirely surprising since, for reasons not understood, both IgA and IgG subclasses exhibit restrictions in their specificity to various types of antigens (Mestecky et al., 1986). It is believed that the character of the antigen (i.e., carbohydrate versus protein) influences the generation of such subclass-restricted responses. However, evidence suggests that other factors are involved; S-IgA antibodies directed against polysaccharide antigens are predominantly found in the IgA2 subclass, while those in sera are manifested as IgA1

(Brown et al., 1985b). Considering the differences between IgA subclass specificities generated in the systemic and mucosal compartments, it may be noteworthy that both serum and S-IgA of infected individuals appear to react almost exclusively against HIV *env* proteins (Kozlowski & Jackson, 1992; Archibald et al., 1987a, 1987b; Janoff et al., 1994). Although our results with colostral IgA must be interpreted with caution because of the small sample size, we have found a similar restriction of the S-IgA anti-HIV response to the IgA1 subclass.

FUNCTION OF IgA IN HIV INFECTION

Neutralization of HIV Infection by IgA. Data from our laboratory indicate that antigen-specific IgA antibodies could potentially play a protective role in HIV infection by participating with monocytes in ADCC reactions against HIV-infected cells (Black & Jackson, 1994) and in neutralization of virus (Kozlowski et al., 1994). We have found that IgA antibodies recognizing the gp120 V3 loop are generated in HIV-infected individuals. The proportions of V3 antibodies and total HIV antibodies in serum IgA (and two colostral IgA samples) were significantly lower than IgG of similar specificity. Production of IgA, however, on a daily basis in bone marrow of normal individuals approximates that of IgG (Mestecky et al., 1986). Concentrations of IgA in normal sera are lower than those of IgG as a result of the shorter half-life of IgA (Mestecky et al., 1986). Therefore, it is possible that synthesis of IgA HIV antibodies in infected individuals is actually equal to that of IgG HIV antibodies. Immunization with vaccines containing the V3 loop could potentially induce a higher level of HIV type-specific IgA antibodies than those produced in infected individuals.

Our results also suggest that IgA antibodies recognizing group-specific determinants of HIV are produced by infected individuals and are responsible for neutralizing HIV-1_{IIIB} infection of cells *in vitro*. Others have recently demonstrated that IgA antibodies of infected individuals can neutralize HIV-1_{MN} infection of CEM T cells (Burnett et al., 1994). Although it is not clear whether neutralization in this study was

mediated by group-specifc or type-specific antibodies, IgA of these subjects was shown to react against both $gp120_{MN}$ and the HIV-1_{MN} V3 loop.

Enhancement of HIV Infection by IgA. The above studies suggest that HIV vaccines can be designed specifically to elicit type-specific V3 or group-specific HIV neutralizing IgA antibodies. However, our subsequent analyses suggest IgA antibodies produced by vaccinated individuals should be examined for potential both to neutralize and to expedite HIV infection of cells. We have found that IgA from sera of infected individuals can mediate ADE of HIV infection in the U937 cell line through the FcαR. When compared to IgG, twice as many individuals (70%) in this study population had IgA that functioned to enhance HIV infection, a phenomenon most prevalent in IgA from ASY individuals. Although our results suggest that physiological concentrations of IgG may inhibit IgA-mediated ADE in sera, it is possible that enhanced HIV infection could occur at mucosal sites where IgA concentrations are generally greater than those of IgG (Underdown & Mestecky, 1994). S-IgA could also function to enhance HIV infection of cells with IgA receptors in the mucosal environment. We have tested IgA purified from colostrum of two HIV-infected women and discovered that both of these IgA samples enhanced HIV infection of U937 cells.

Although enhancing epitopes on HIV have been reported to reside within highly conserved regions of gp41 (Eaton et al., 1994; Robinson et al., 1991), data have also been presented suggesting that nonneutralizing antibodies exhibiting weak cross-reactivity with the V3 loop can mediate enhancement, while those strongly cross-reactive neutralize (Jiang et al., 1991). We were unable to demonstrate any relationship, however, between concentrations of HIV-1_{IIIB} V3 antibodies in purified IgA or IgG samples and ability to enhance HIV-1_{IIIB} infection in U937 cells. Although a higher incidence of enhancing IgA in sera of ASY individuals was found, concentrations of HIV-1_{IIIB} V3 IgA antibodies were not significantly different from those in AIDS patients. One difference that we have noted in our studies between IgA of ASY and AIDS patients in regard to

HIV reactivity is that ASY IgA is generally more effective at neutralizing infection of CEM T cells via group-specific determinants. If this observation is related to the ability of IgA to enhance infection, it suggests the presence of higher concentrations of IgA antibodies recognizing a conserved region of HIV mediate enhancement. The fact that IgA purified from sera of individuals participating in this study has the capability to enhance HIV-1_{IIIB} infection of cells supports the concept that antibodies recognizing a conserved determinant mediate ADE, since this isolate is extremely uncommon within HIV-infected individuals in North America (LaRosa et al., 1990).

As discussed in the third paper of this work, more effective neutralization and the higher prevalence of HIV infection enhancement phenomena in ASY IgA could be associated with the presence of more polymeric IgA1 HIV antibodies in IgA samples from these individuals, as compared to those with AIDS. In view of the fact that the vast majority of IgA synthesized by plasma cells in the lamina propria or interstitial regions is polymeric, an ability of polymeric HIV antibodies to more effectively participate in ADE could have profound ramifications in the development of a mucosal HIV vaccine..

Complement and HIV antibodies are believed to function to enhance HIV infection through increasing uptake of virions and proviral formation in cells. Our results suggest, however, that enhanced HIV infection may also be related to signals transduced following endocytosis of FcR and ligand. High concentrations of non-HIV-specific SN IgA could stimulate HIV production in U937 cells via binding to the FcαR. It is quite possible that cross-linking of FcgR or FcαR on U937 induces production of a cytokine such as TNF-α, which up-regulates HIV replication. Since the magnitude of enhancement by SN IgA was considerably below that obtained with SP IgA, the presence of HIV-specific antibodies still appears to be necessary for demonstrating significant infection enhancement. The mechanism of ADE of HIV infection may involve a combination of HIV antibodies which facilitate uptake of HIV virions and production of soluble factors that accelerate viral replication. This hypothesis could be tested by

measuring concentrations of TNF-α or other cytokines in culture medium of U937 cells infected with HIV in the presence or absence of IgA from SN and SP individuals. If TNF-α is directly involved in ADE, the addition of an anti-TNF-α antibody to cultures should abrogate or reduce enhanced infection by IgA.

ADE of Emerging HIV Variants. The potential significance of ADE in relation to expansion in HIV-infected individuals of neutralization-resistant viral variants has been underappreciated. It is now known that macrophage-tropic, non-synctium-inducing (NSI) HIV isolates are the first to appear in infected individuals, despite the wide array of HIV isolates having a T-cell tropic, synctium-inducing (SI) phenotype in the transmitting individual (Zhu et al., 1993). Sequence variation in the *env* gene of the transmitted isolate, termed the "major species," occurs in as little as 2 weeks after transmission (Pang et al., 1992). The emergence of these early viral variants, the "minor species," is not solely related to selection pressures exerted by the host immune system (Pang et al., 1992) but is partly a reflection of the hypermutability associated with HIV replication (Preston et al., 1988). In one individual, 9 detectable minor genotypic variants were found to develop in as few as 5 days. In one month, this number increased to 16 (Pang et al., 1992). Minor and major variant genomes within an individual have been found to vary by as much as 33% (Saag et al., 1988). This population of related but distinct genotypic variants has been termed the "quasispecies" (Goodenow et al., 1989). Even greater diversity exists between the genotypic character of the quasispecies in one individual and that in other individuals (Saag et al., 1988).

The rapid generation of viral variants continues throughout HIV infection, with fluctuations in predominating species (Meyerhans et al., 1989; Goodenow et al., 1989). Expansion of minor species has been speculated to be related to selection by neutralizing antibodies against the major species and to differences in cell tropism of variants (Fisher et al., 1988). Although early viral species in infected individuals are NSI, cytopathic variants with SI phenotypes begin to emerge over the course of disease and eventually

predominate (von Gegerfelt et al., 1991; Cheng-Mayer et al., 1988; Tersmette et al., 1989). Conversion from macrophage-tropic NSI to the T cell-tropic SI phenotype is associated with increased virulence of HIV; emergence of these rapidly growing SI variants in infected individuals correlates with declines in $CD4^+$ count and a poor prognosis (Asjö et al., 1986; von Briesen et al., 1987; Tersmette et al., 1989).

It is possible that antibodies mediating enhancement, rather than neutralization, could facilitate the expansion of HIV minor species with more diverse V3 loops. It will be difficult to resolve many issues concerning the contribution of neutralizing and enhancing antibodies to this disease *in vivo*. For instance, HIV "homotypic" isolates derived from sequential passaging in PBMC differ considerably from quasispecies existing *in vivo* (Meyerhans et al., 1989). Two predominant *in vivo* HIV species of one individual were not detected after PBMC were cocultured with normal PBMC. Instead, the major viral species in these cultures was an *in vivo* minor variant (Meyerhans et al., 1989). Therefore, studies using autologous sera or purified Ig and homotypic isolates may not accurately reflect enhancement *in vivo* or correlate with pathogenesis of HIV infection.

IgA-Mediated ADE of HIV Infection in the Mucosa. The ability of IgA to enhance HIV infection could have profound consequences in mucosal regions such as GALT, where large numbers of potentially HIV-infectable macrophages and lymphocytes reside. Mucosal macrophages express the FcαR (Shen, 1992) and may be susceptible to enhanced infection in the presence of locally produced HIV-specific pIgA antibodies complexed with virus. The formation of such immune complexes could also potentially lead to infection or enhance infection of pIgR-expressing epithelial cells. Although our attempts to infect the human endometrial-derived $pIgR^+$ HEC epithelial cell line with $HIV\text{-}1_{IIIB}$ or the monocyte-tropic $HIV\text{-}1_{Ba\text{-}L}$ in the presence of pIgA from infected individuals have been unsuccessful, we do have preliminary evidence that HIV infection of $pIgR^+$ HT-29 colonic carcinoma cells can be enhanced in the presence of IgA from

sera of infected individuals. However, both mIgA and pIgA were found to be capable of mediating this phenomenon. Therefore, another IgA receptor expressed by this cell line may participate in enhancement. The pIgR$^+$ T84 colon carcinoma may also be useful in studies of IgA-mediated ADE of HIV infection in epithelial cells. Our studies have indicated that a factor in seropositive sera, presumably IgA or IgM, which binds to these cells can lead to enhanced HIV infection of this cell line.

M cells in the mucosal epithelium have been proposed to play a role in sexually acquired HIV infection in the rectum by serving as a port of entry for HIV into the mucosa (Amerongen et al., 1991). Some pathogens such as reovirus, *Salmonella typhi*, *Shigella flexneri*, and *Yersinia enterocolitica* are internalized by M cells but manage to colonize neighboring epithelial or lymphoid cells where they proliferate and disseminate systemically before a sufficient mucosal immune response is generated (Kraehenbuhl & Neutra, 1992). HIV has been shown to adhere to M cells using PP explants from mice and rabbits (Amerongen et al., 1991). Subsequent endocytosis and delivery of HIV virions to intraepithelial lymphocytes and macrophages in these M cells have also been demonstrated in electron micrographs (Amerongen et al., 1991).

It remains to be determined whether HIV is bound and internalized by human M cells or can productively infect M cell-associated immune cells. These cells could enhance viral infection via uptake of HIV/S-IgA complexes from the lumen since they exhibit IgA binding capability (Weltzin et al., 1989). In addition, M cell binding of non-HIV-specific V_H3-expressing S-IgA antibodies complexed with virions through interaction with gp120 could also theoretically facilitate enhanced entry of HIV into the mucosa and profoundly contribute to the dissemination of virus within the host.

The significance of enhancing antibodies in regard to the pathogenesis of HIV infection has perhaps been diminished by contrasting the much lower 2-fold magnitude of enhancement observed *in vitro* for HIV isolates with the 10- to 100-fold magnitude of enhancement associated with dengue viruses (Porterfield, 1986). However, unlike HIV,

dengue infections are not chronic in nature. ADE-mediated 2-fold increases in viral replication could profoundly contribute to the pathogenesis of HIV infection over the protracted course of this disease. Further, this phenomenon could be directly related to the expansion of neutralization-resistant viral variants and the emergence of cytopathic SI variants. In conclusion, the potential for ADE to occur *in vivo* and its possible relevance in regard to the failure of the HIV-driven humoral immune response to eradicate virus should not be discounted. The problems recently encountered in vaccine trials emphasize the need for further study and understanding of this phenomenon so that prevention, rather than facilitation, of disease in immunized individuals later exposed to HIV can be achieved.

Future studies should address the possibility that IgA-mediated ADE of HIV infection in FcαR$^+$ Kupffer cells, ASGP-R$^+$ hepatocytes, and pIgR+ epithelial cells may contribute to HIV pathogenesis. In particular, polymeric and S-IgA in both infected individuals and vaccine recipients should be examined for HIV infection enhancing capability. Epitopes of HIV recognized by IgA antibodies which enhance HIV infection should also be elucidated so that these determinants can be excluded from potential vaccines. It may also prove beneficial to investigate potential mediators of HIV-related IgA B cell polyclonal activation so that vaccines can be designed to induce antigen-specific, rather than nonspecific, responses.

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APPENDIX

A POLARIZED HUMAN ENDOMETRIAL CELL LINE WHICH BINDS AND TRANSPORTS POLYMERIC IgA

A POLARIZED HUMAN ENDOMETRIAL CELL LINE WHICH BINDS AND TRANSPORTS POLYMERIC IgA

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Summary

We have demonstrated that a human endometrial cell line, HEC-1, maintains a high transepithelial electrical resistance, directionally transports fluids across the cell monolayer, and releases enveloped viruses at distinct plasma membrane domains: influenza virus is released at the apical surfaces and vesicular stomatitis virus at the basolateral surfaces. In addition, we have examined the expression of domain-specific endogenous proteins, including the polymeric immunoglobulin receptor. Multiple endogenous polypeptides were found to be secreted into the culture medium at basolateral surfaces, whereas no secretion of specific polypeptides was observed from apical cell surfaces. Distinct patterns of endogenous proteins were also observed on apical and basolateral cell surfaces, with a much more complex polypeptide pattern on the basolateral membranes. Using surface biotinylation and immunofluorescence, the polymeric immunoglobulin receptor was found to be expressed on the basolateral surfaces of HEC-1 monolayers. The specific binding of polymeric IgA was found to occur on the basolateral surface and was followed by transcytosis to the apical surface and release into the apical medium. The observed characteristics indicate that the endometrium-derived HEC-1 epithelial cell line can be employed as a model for studies of protein transport in polarized epithelial cells of human endometrial tissues, as well as for studies of the interaction of microorganisms with epithelial cells in the genital tract.

Introduction

Polarized epithelial cells are distinguished morphologically by apical projections or microvilli, tripartite junctional complexes, and a defined basal membrane which is contiguous with the lateral plasma membrane (11). A number of continuous epithelial cell lines retain in culture the structural and functional properties of cells in epithelial tissues (20,58). Transepithelial transport properties are also retained by cells in culture as evidenced by the formation of domes, which are the result of apical to basolateral transport

of solutes and fluids that remain trapped between the cell monolayer and the fluid-impermeable culture dish (23). When grown on permeable membrane supports, polarized epithelial cell monolayers develop a high transepithelial electrical resistance due to the presence of occluding junctions which form a selective ionic barrier (10,14,36). In addition to limiting the diffusion of molecules across the cell monolayer, the tight junctions divide the plasma membrane into two distinct domains which differ in their protein and lipid compositions (20,54). The pathways which regulate intracellular sorting and the asymmetric delivery of plasma membrane proteins to the cell surface have been found to vary among epithelial cell types and the tissues from which they are derived (48,55,66). Because of the functional similarity of many epithelial cell lines to the cells in the tissues from which they were derived, such cells have provided useful models for the study of protein traffic as well as the interaction of microorganisms with epithelial cell surfaces.

Cells from human endometrial adenocarcinomas have been grown in culture and used for the investigation of endometrial tumor cell growth and metabolism (12,26,45,60). Cells derived from human endometrial tissues and grown on an extracellular matrix appeared as polygonal cells covered with microvilli (6). Others (44) have reported that the *in vitro* growth of human endometrial cells resulted in the formation of glandular structures consisting of columnar epithelium surrounding a central lumen. Continuous cell lines from human endometrial carcinomas have been established and the substrate requirements, karyotopic and cytogenetic features, cytological/histochemical and immunological characteristics, and estrogen binding properties have been investigated (13,26,32,41). The human endometrial cancer-one (HEC-1) continuous cell line was established by Kuramoto et al. (26) from a papillary adenocarcinoma of human endometrium. In the present study, we have characterized the HEC-1 cell line with respect to epithelial polarity.

The finding that enveloped viruses mature by budding at specific plasma membrane domains in polarized epithelial cells (48) has led to many studies of virus-infected epithelial cells in which directional transport and surface expression of virus-specific proteins, as

well as virus entry and release at specific plasma membrane domains, have been investigated (for review, see ref. 61). We have examined the release of enveloped viruses in HEC-1 cells to determine the ability of these cells to effect directional transport of surface proteins. The surface-specific expression of endogenous proteins and the pattern of release of secretory proteins have also been investigated.

The role of the polymeric immunoglobulin (pIg) receptor in the delivery of polymeric immunoglobulins IgA and IgM to mucosal surfaces is critical for immune protection against pathogenic organisms (35,62). The pIg receptor binds the immunoglobulin ligand on the basolateral surface of epithelial cells which line glandular and mucosal surfaces. This receptor-ligand complex is then transported through vesicular compartments to the apical surface, where cleavage of the pIg receptor results in the release of the pIg complexed to the extracellular domain of the receptor, called secretory component (SC) (8,34,39,53). Thus, antibodies secreted in the interstitial space by local plasma cells reach mucosal surfaces via the pIg receptor. Many epithelial cells and rodent hepatocytes express pIg receptors which bind the pIg's IgA and IgM on the basolateral surface, transport the immunoglobulin across the cell, and release the ligand at the apical surface (8,22). We have also investigated the potential of HEC-1 cells to effect polarized transport of polymeric IgA (pIgA), which may be relevant to the immunology of the human genital tract.

Materials and Methods

Cells and Viruses

HEC-1 cells were the gift of Dr. A. Menge and were grown in modified RPMI 1640 medium (ICN Biomedicals, Inc., Costa Mesa, CA) supplemented with 9% fetal bovine serum (FBS). Madin-Darby canine kidney (MDCK), Madin-Darby bovine kidney (MDBK), and baby hamster kidney cells (BHK-21) were obtained from the American Type Culture Collection and maintained in Dulbecco's Modified Eagles (DME) medium supplemented with 3% FBS and 6% bovine calf serum (Hyclone Lab., Inc., Logan, UT).

All cells were assayed for mycoplasma by DNA fluorochrome staining (29) and found to be mycoplasma free. For phase-contrast and electron microscopy studies, the cells were plated on 35 mm plastic dishes and cultivated until the presence of domes was evident (7-10 days post seeding). For analysis of protein expression and virus release, cells were grown on Millicell-HA tissue culture inserts (0.45 μm, 4.5 cm^2, Millipore Corp., Bedford, MA) and monitored for transepithelial electrical resistance (see below).

Stocks of the A/WSN/33 (H1N1) strain of influenza virus were grown in MDCK cells and titered by plaque assay in MDCK cells by a modification (50) of the procedure of Tobita et al. (59). The Indiana strain of vesicular stomatitis virus (VSV) was grown and titrated by plaque assay in BHK-21 cells. VSV-infected monolayers were overlaid with DME supplemented with 2% low-melt agar and 1% FBS, incubated at 37°C for 2 days, fixed with 10% formaldehyde, and stained with 20% crystal violet solution.

For viral infection, HEC-1 monolayers were washed twice with phosphate-buffered saline (PBS) and inoculated with either VSV or influenza virus in RPMI 1640 medium at a multiplicity of 4-8 plaque-forming units (pfu) per cell. Initial experiments tested the efficiency of viral entry on apical and basolateral surfaces of filter-grown HEC-1 monolayers. Virus was added to each surface and absorbed for 1 h at 37°C. Cells were metabolically labeled for 4-6 h and examined for virus-specific proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. The results revealed that influenza virus preferentially entered the HEC-1 cells at apical surfaces, whereas VSV preferentially infected at basolateral surfaces. All subsequent infections on the filter-grown cells were performed accordingly. Following the 1 h absorption period, the inoculum was removed, the monolayer rinsed with PBS to remove unabsorbed virus, fresh RPMI 1640 medium supplemented with 2% FBS added, and incubation continued for 4 to 18 h.

Electrical Resistance Measurements

HEC-1 monolayer integrity was monitored by measuring transepithelial electrical resistance using a Millicell-Electrical Resistance System (ERS) (Millipore, Bedford, MA) according to the manufacturer's operating instructions. All resistance measurements were made across confluent cell monolayers grown on membrane inserts, which were placed in medium lacking bovine serum at room temperature. Resistance values of blank filters were subtracted from each reading.

Electron Microscopy

HEC-1 cell monolayers were fixed with buffered 1% glutaraldehyde for 30 min at 6, 8, 10, and 12 h post infection (hpi). The cells were post-fixed for 1 h with 1% osmium tetroxide, dehydrated with a graded ethanol series, and embedded in electron microscopy (EM) bed 812 (Electron Microscopy Sciences, Ft. Washington, PA). Thin sections were prepared on a Reichert ultramicrotome, mounted on 300 mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a Phillips EM301 electron microscope.

Metabolic Labeling

The medium from both compartments of the filter inserts was replaced with DME lacking methionine and bovine serum and incubated for 45-60 min at 37°C. Following the methionine starve, 150-200 μCi/ml of [^{35}S]-methionine/cysteine (DuPont NEN, Boston, MA) was added to the basolateral medium and the incubation was continued for various intervals.

Virus Release

To determine the kinetics of release of radiolabeled virus particles, confluent HEC-1 cell monolayers on permeable filter supports were infected with VSV or influenza virus, and metabolically labeled for 6 h beginning immediately after the absorption period or at 2, 4, 6, and 11 hpi. The apical and basolateral medium was removed and clarified at 300 x g for 5 min. The supernatants were transferred, reclarified at 14,000 x g for 10 min, and overlaid on a 25% sucrose cushion. Radiolabeled virus which was released into the

medium was pelleted by centrifugation at 100,000 x g for 90 min. The supernatants were discarded and the viral pellets were suspended in SDS-PAGE sample buffer, boiled for 3 min, and analyzed by SDS-PAGE (12% acrylamide under reducing conditions) and fluorography.

To determine the time course of release of infectious virus, duplicate filters were infected with VSV or influenza virus as above. At 5, 7, 9, 11, 13, and 18 hpi, the medium from the apical and basolateral compartments was removed and clarified at 300 x g for 5 min at 4°C, and infectious virus yields were determined by plaque assay.

Biotinylation of Cell Surface Proteins

HEC-1 cells were plated on permeable filter supports, grown until transepithelial electrical resistance was above 300 ohms x cm^2, and labeled with [^{35}S]-methionine for 6 h. To remove serum proteins, the apical and basolateral surfaces were washed four times with cold PBS supplemented with 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ (PBS-CM). Surface-specific biotinylation was accomplished by the addition of 1 ml of 0.5 mg/ml NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) to one or the other compartments and allowing the biotin reagent to react for 30 min at 4°C on a rotating platform (17,52). One milliliter of cold PBS-CM was added to the opposite compartment. NHS-SS-biotin was prepared as a 200 mg/ml stock in DMSO and diluted in PBS-CM immediately before use. Free biotin was removed and quenched by washing both surfaces twice with RPMI 1640 for 2 min and twice with PBS-CM. The membrane inserts were excised with a razor blade; covered with lysis buffer (150 mM NaCl, 1% NP40, 1% DOC, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 1 μg/ml E-64, and 2 μg/ml aprotinin (Sigma Chemical Co., St. Louis, MO); and incubated at 4°C for 30-60 min. The lysates were clarified by centrifugation (14,000 x g) for 10 min at 4°C. To separate the biotinylated surface proteins, the cell lysates were reacted with 50 μl of a 50% suspension of streptavidin-agarose (Pierce) for a minimum of 5 h at 4°C. After binding, the immobilized streptavidin was recovered by centrifugation (14,000 x g for 5 min) and the

agarose beads were washed four times in lysis buffer. Bound proteins were eluted by suspension in SDS-PAGE sample buffer, heated at 100°C for 5 min, separated by SDS-PAGE under reducing conditions (10% or 12% acrylamide gels), and visualized by fluorography. Results of surface biotinylations were reproduced a minimum of 3 times.

Binding and Transcytosis of pIgA

Human monomeric immunoglobulin A (mIgA) and pIgA were isolated from sera obtained from myeloma patients as previously described (33) and radioiodinated by the chloramine T procedure (19). Free iodine was removed on a Sephadex G-25 (Pharmacia LKB, Piscataway, NJ) column and the specific activity was estimated to be 4.2×10^6 cpm/μg for mIgA and 4.4×10^6 cpm/μg for pIgA.

Ten micrograms of radioiodinated pIgA or mIgA were added to the basolateral medium of HEC-1 cell monolayers grown on permeable membrane supports and incubated for 3 h on ice. The medium from both surfaces was removed, cell surfaces were washed twice with PBS, and fresh medium was added. The inserts were placed at 37°C and incubated for 90 min. The medium from the apical compartment was removed, precipitated with 10% TCA (C_f), and counted in a Beckman 4000 gamma counter (Beckman, Fullerton, CA).

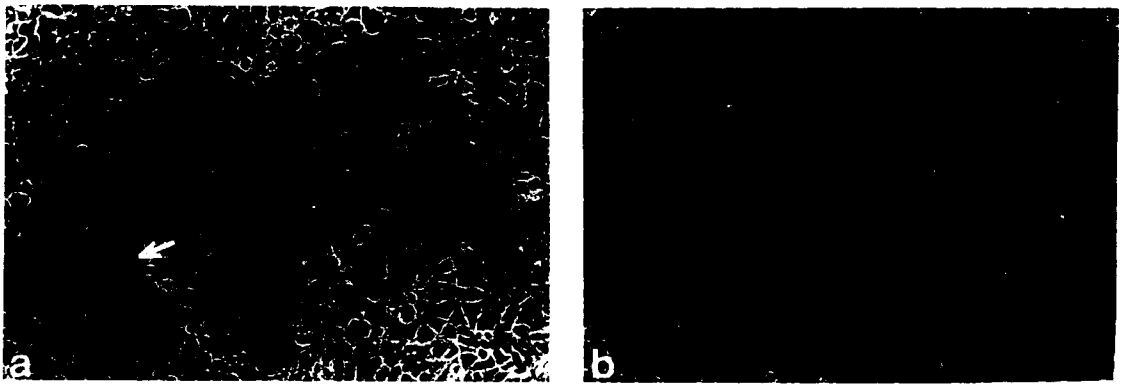
To determine the kinetics of pIgA transcytosis, replicate filters of polarized monolayers of HEC-1 cells were incubated with 3.6 μg of radiolabeled mIgA or pIgA (8 filters for each molecular form of IgA) for 3 h on ice (as above) and then transferred to 37°C. The medium was harvested after 1, 2, 3, and 4 h, TCA precipitated, and counted in a gamma counter.

Results

The HEC-1 Cell Line Exhibits a Polarized Epithelial Phenotype

Polarized epithelial cell layers exhibit structural and functional properties which enable these cells to transport ions and fluids across the cell monolayer, which results in formation of fluid-filled domes that can be visualized by phase-contrast microscopy

FIG 1. Phase-contrast micrographs of HEC-1 monolayers at (a) 24 h and (b) 4 days after the cells reached confluency. The characteristic cobblestone appearance of an epithelial cell layer is evident. Initial dome formation was observed at 24 h (arrow); the domes then continued to grow in size with additional time of incubation.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b

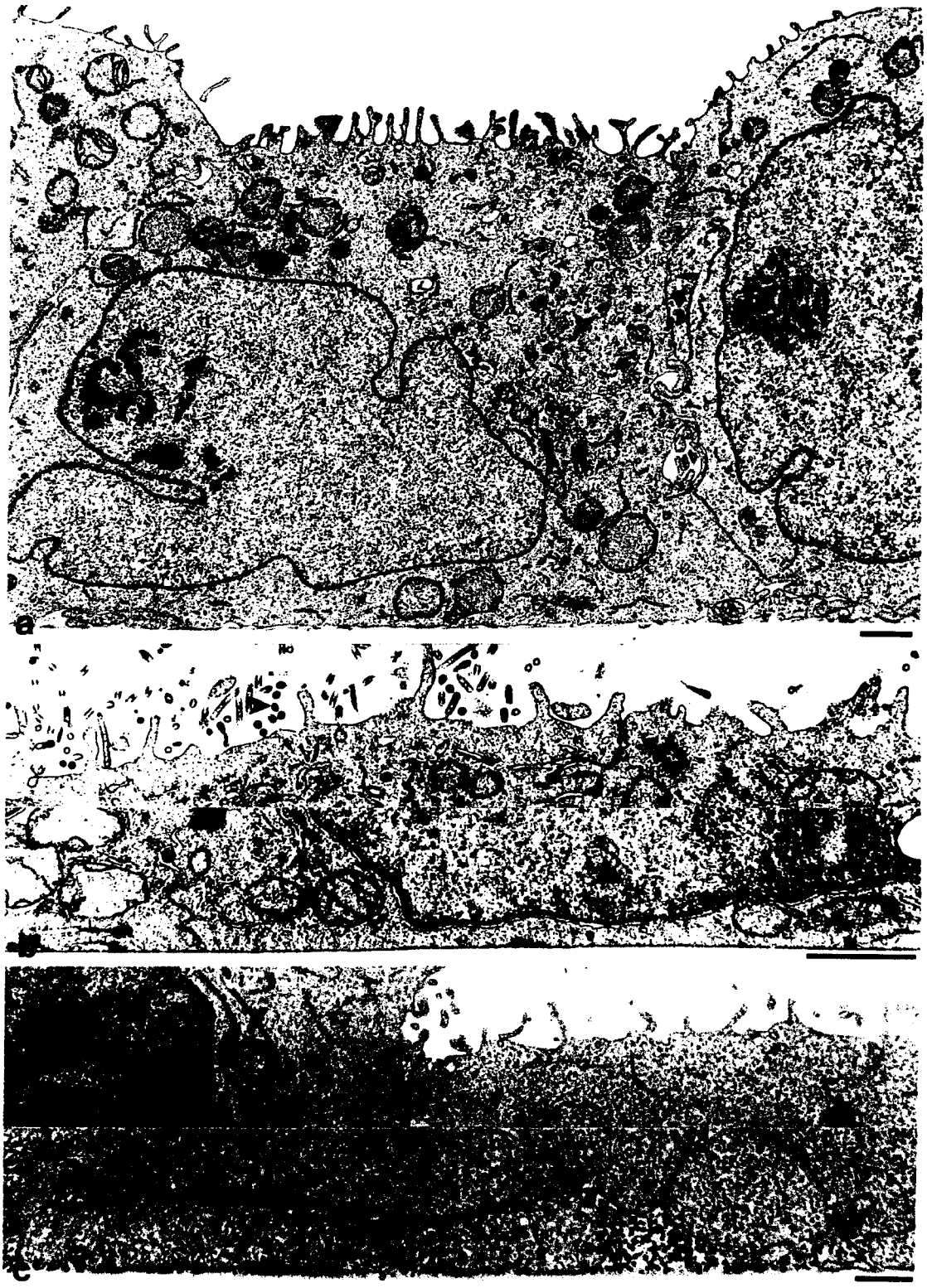
(23,28). HEC-1 cell monolayers exhibited a characteristic cobblestone appearance. In addition, large domes or fluid-filled blisters were observed throughout the monolayer (Fig. 1). Initial dome formation was observed about 24 h after the cells reached confluency (Fig. 1a), and the appearance of the domes was more pronounced several days after confluency (Fig. 1b). The formation of domes indicates that the HEC-1 cells form monolayers which exhibit the ability to transport fluids across the cell layers.

To further compare the HEC-1 cell line to other polarized epithelial cell lines, HEC-1 cells were grown on permeable membrane supports and transepithelial electrical resistance was measured across the confluent monolayer. Similar to the MDCK cell line, the establishment of cell surface polarity was gradual, requiring a minimum of 60 h of culture before a high transepithelial electrical resistance was established (2). Following 24 h of culture, the transepithelial electrical resistance was consistently below 100 ohms x cm^2 and increased to 125-150 ohms x cm^2 by 48 h after seeding at a density of about 4 x 10^5 cells/well. Subsequently, there was a dramatic rise in transepithelial electrical resistance concomitant with the establishment of extensive cell-cell contacts and junctional complexes. The resistance measurements reached levels greater than 400 ohms x cm^2 at 4-6 days post seeding. Thus, HEC-1 cells establish a high transepithelial resistance, as is characteristic of polarized epithelial cell monolayers.

Asymmetric Budding of Enveloped Viruses

The assembly of enveloped viruses has been used frequently to investigate the pattern of transport of membrane proteins and to demonstrate the polarity of epithelial cells. To determine if enveloped viruses mature at distinct plasma membrane domains in the HEC-1 cells, monolayers were infected with VSV or influenza virus and the site of maturation was examined. The HEC-1 cells exhibited morphological properties characteristic of a polarized phenotype, including apical microvilli, junctional complexes, a distinguishable basal membrane, and lateral contacts with neighboring cells (Fig. 2, panel a). Influenza virus was found to mature exclusively at the apical surfaces; no virus

FIG 2. Assembly of enveloped viruses in HEC-1 cells. Uninfected cells (panel a) show apical microvilli and junctional complexes characteristic of polarized epithelial cells. HEC-1 cells were infected with either influenza virus (panel b) or VSV (panel c) at a multiplicity of 5. At 6 h (influenza) or 8 h (VSV) post infection, the cells were washed and fixed with 1% glutaraldehyde, post fixed with 1% osmium tetroxide, and stained with uranyl acetate and lead citrate. Influenza virus (arrowhead, panel b) is localized at the apical surface, while the basolateral surface remains free of virus. VSV is released at the basolateral surface (arrow); no virus particles are seen at the apical surface.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budding was observed along the basolateral membranes (Fig. 2, panel b). In contrast, VSV virions were only detected at the basolateral surfaces; the apical membranes remained virus free (Fig. 2, panel c). These results demonstrate that the two enveloped viruses, influenza virus and VSV, are assembled and released at specific plasma membrane domains in infected HEC-1 cells. Hence, the HEC-1 cells exhibit the same pattern of polarized maturation of these enveloped viruses which was reported previously for cell lines originating from kidney or intestinal epithelium (46,48).

The asymmetric release of VSV and influenza virus in HEC-1 cells was quantitated by analyzing the apical and basolateral medium for the presence of metabolically labeled virus proteins during the time course of infection. As seen from the pattern of radiolabeled, virus-specific proteins in Fig. 3, influenza virus was released exclusively into the apical medium at 9, 11, and 13 hpi. No virus or viral proteins were recovered at 5 and 7 hpi. By 18 hpi, low levels of influenza virus proteins were also observed in the basolateral medium (not shown). It is likely that the presence of influenza virus particles in the basolateral medium at this time is due to virus-induced disruption of the tight junctions, which would interrupt cell polarity and allow the virus to diffuse into the basolateral medium. This conclusion was supported by the observation that a concurrent loss of transepithelial electrical resistance was observed at 18 hpi when HEC-1 cells were grown on permeable membranes and infected with influenza virus and by the appearance of cytopathic effects (cpe) in the cell monolayer at 18 hpi. In contrast to the apical release of influenza virus, the release of VSV was demonstrated to occur predominantly into the basolateral medium at 13 and 18 hpi by the progressively increasing amounts of radiolabeled virus-specific proteins found in the basolateral medium.

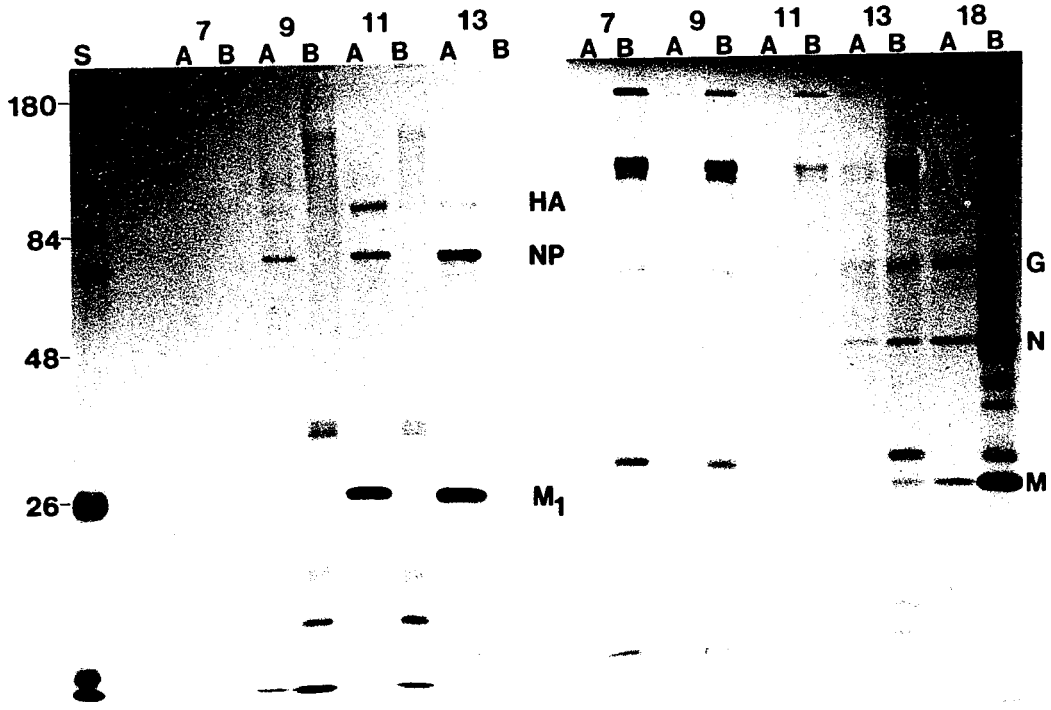
To demonstrate the release of infectious virus, the medium from the apical and basolateral compartments of infected, filter-grown HEC-1 cells was analyzed by plaque assay. Fig. 4a shows that infectious influenza virus was detectable in the apical medium by 5 hpi, and the titer continued to rise progressively with time, reaching a titer of 2×10^7

pfu/ml at 13 hpi. No influenza virus was detected in the basolateral medium at 5, 7, 9, or 11 hpi, and at 13 hpi the basolateral medium showed titers over 1000-fold lower than the corresponding apical compartment. In contrast, infectious VSV particles were preferentially released into the basolateral medium (Fig. 4b). Infectious virus was detected in the basolateral medium by 7 hpi. At 9 or 11 hpi, 99% of the infectious VSV was found in the basolateral medium; at 13 and 24 hpi, 91%-92% of the infectious virus was recovered in the basolateral medium. These quantitative results demonstrate that the release of influenza virus and VSV occurs at specific membrane domains, as previously reported in other polarized cell types.

Polarized Expression of Endogenous Proteins

During the development of a polarized phenotype, the distribution of membrane proteins becomes restricted to specific membrane domains (1,65). In addition, an asymmetric secretion of endogenous proteins has been reported in several epithelial cell lines (18,46,63). We therefore compared the proteins expressed on the apical and basolateral surfaces of polarized HEC-1 cells grown on permeable membrane supports, as well as the proteins secreted into the culture media. In addition to illustrating the polarized release of enveloped viruses, Fig. 3 reveals that several radiolabeled proteins were secreted into the basolateral medium. Two distinct, small polypeptides (apparent Mr below 26 kD), a doublet at approximately 37 kD, and a large polypeptide at about 120 kD were evident in the basolateral medium of influenza virus-infected cells (Fig. 3). Similarly, the basolateral medium of the VSV infected HEC-1 cells shows the same two small protein bands and the band at approximately 120 kD. Similar proteins were observed by direct analysis of the basolateral medium of uninfected HEC-1 cells when precipitated with either TCA (not shown) or methanol. Methanol precipitation of the basolateral medium (Fig. 5) shows several bands below 26 kD, a sharp band at about 53 kD, and multiple proteins in the range of 80-180 kD. These proteins were observed in the media beginning about 3 h after radiolabeling. In contrast, no endogenous protein bands were detected in the apical

FIG 3. Directional release of influenza virions (left panel) and VSV (right panel). Cells were grown on permeable supports, infected and radiolabeled as described in Materials and Methods, and at the times indicated the apical (A) and basolateral (B) media were harvested (7-18 h). The influenza virus major structural proteins, hemagglutinin (HA), nucleoprotein (NP), and matrix protein (M1) are indicated in the standard (S); VSV-specific radiolabeled proteins are shown on the right (G, N, M); and electrophoretic mobilities of prestained molecular weight standards are indicated on the left. The influenza structural proteins are present in the apical medium, whereas VSV structural proteins are predominantly observed in the basolateral medium. A number of endogenous proteins are seen in the basolateral medium, but not in the apical medium. Similar endogenous proteins were observed following TCA and methanol precipitation.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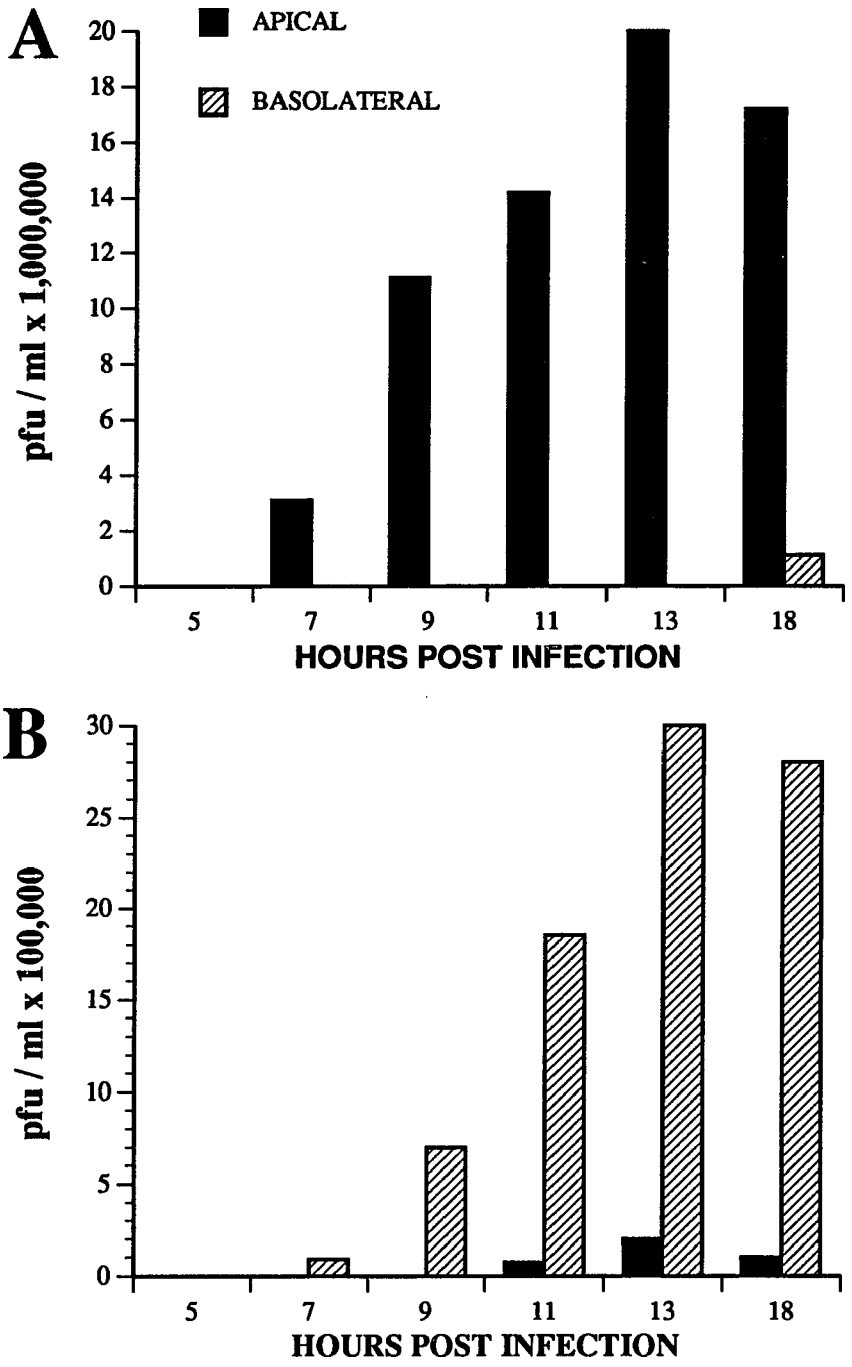
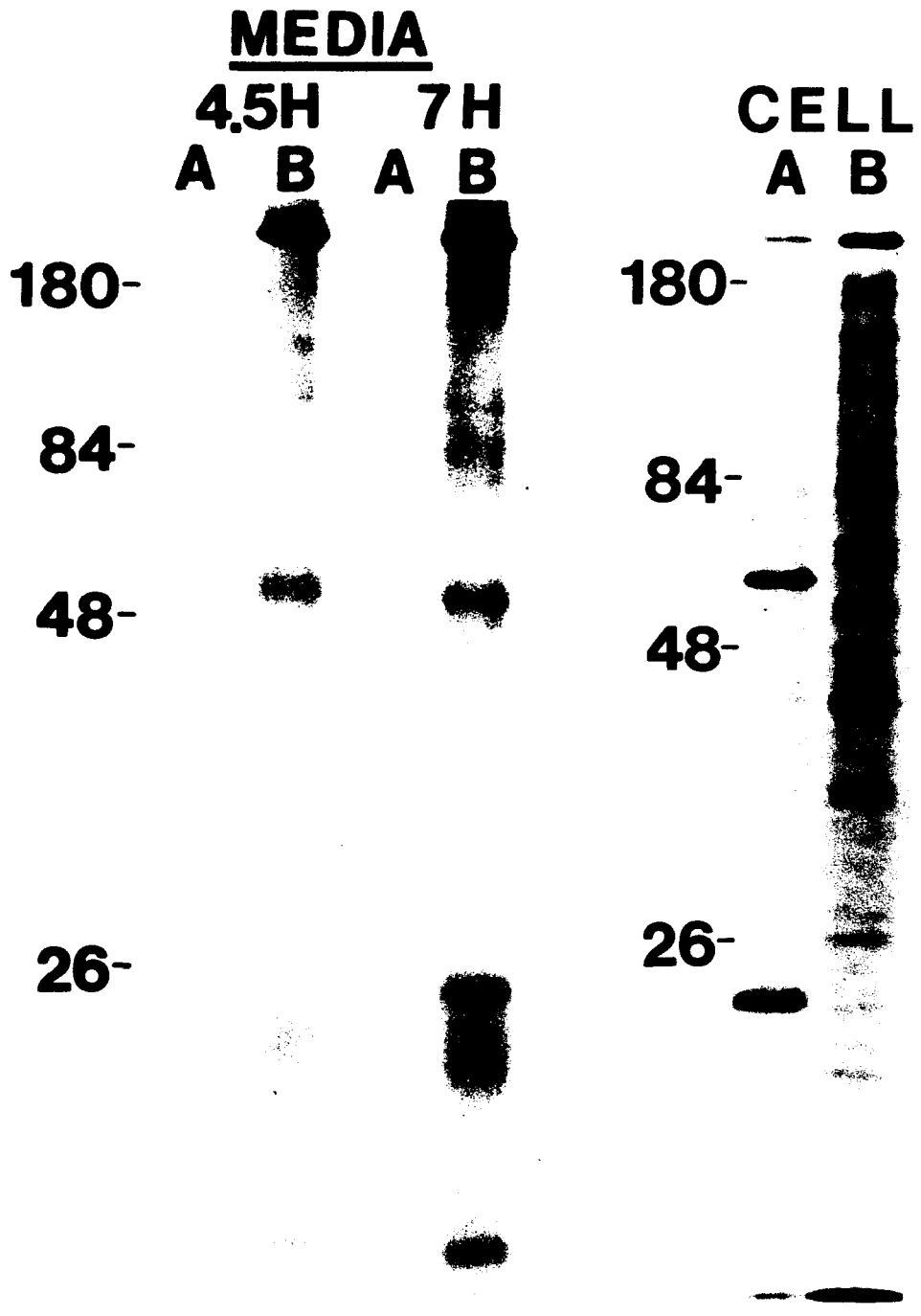


FIG. 4. Infectious virus yields from HEC-1 cells grown on filter inserts. Cells were infected as described in Materials and Methods, and virus titers were determined by plaque assay of the apical and basolateral media at 5-18 hpi. Infectious influenza virus was released only into the apical medium from 5-13 hpi (panel a). At 18 hpi, virus titers were somewhat reduced and 3.4% of influenza virus was predominantly found in the basolateral medium (panel b).

FIG. 5. Endogenous cell surface and secreted proteins of HEC-1 cells.

Media. Aliquots of the apical and basolateral media were removed after 4.5 h and 7 h, diluted sevenfold in cold methanol, and precipitated overnight at 20° C. The precipitates were pelleted for 15 min at 14,000 x g, air dried, and resuspended in sample reducing buffer. Lanes B show the pattern of proteins secreted into the basolateral medium. No proteins were detected in the apical medium (A).

Cells. HEC-1 monolayers were grown on permeable membrane supports and labeled with [^{35}S]-methionine/cysteine for 6 h, after which each membrane domain was biotinylated as described in Materials and Methods. Radiolabeled cell lysates were precipitated with immobilized streptavidin and examined by SDS-PAGE and fluorography. Distinct patterns of apical (A) and basolateral (B) cell surface proteins are observed.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180-
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medium. Thus, unique endogenous proteins were found to be secreted exclusively into the basolateral medium of HEC-1 cells, a result which is similar to that reported for the polarized Caco-2 intestinal cell line (46).

To compare the pattern of endogenous proteins expressed on the two surfaces of the HEC-1 cell line, cells were metabolically labeled, followed by domain-specific surface biotinylation. A larger number of basolateral surface proteins was detected by this methodology, although several distinct apical surface proteins were also observed (Fig. 5). Two prominent apical surface proteins were evident at approximately 62 and 20 kD (lane A). Multiple bands ranging from about 43 kD to 180 kD, a doublet at about 36 kD, and a band at approximately 29 kD were observed on the basolateral surfaces (lane B). The primary amino groups of the basolateral proteins could be more accessible to the biotinylation reaction, which could play a role in the detection of a larger number of proteins on basolateral surfaces. These results indicate that the major proteins expressed on apical and basolateral surfaces are distinct, consistent with the polarized epithelial phenotype of the cells.

Basolateral Expression of the pIg Receptor

The pIg receptor plays a crucial role in mucosal immunity in that the receptor mediates the transport of polymeric immunoglobulins to mucosal surfaces where they can cross-link or neutralize microbial pathogens (15,31,34). The expression of the pIg receptor on HEC-1 cell surfaces was analyzed by immunofluorescence microscopy and by surface-specific biotinylation, followed by immuno- and streptavidin precipitation. As shown in Fig. 6b, the immunofluorescent staining pattern after treatment of cells with ethylene glycol-bis(ß-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) to open the junctional complexes showed a relatively bright cobblestone appearance, while little apical staining was observed when intact monolayers were examined (Fig. 6d), indicating that the pIg receptor is expressed in HEC-1 cells and is predominantly localized on basolateral membranes. Corresponding data were obtained with HEC-1 cells grown on filter inserts

which exhibited a high transepithelial electrical resistance. Fig. 7 illustrates that the majority of the pIg receptor was localized to basolateral surfaces when cells were analyzed by domain-specific biotinylation and immune precipitation with antibody to secretory component. Based on phospho-imaging analyses it was calculated that 82% of the total pIg receptor found on the surface of polarized HEC-1 cells resides on the basolateral surface domain. These results raised the possibility that HEC-1 cell monolayers were capable of transcytosis of pIgA.

Transcytosis of pIgA

To determine if the basolaterally expressed pIg receptor specifically binds and transports pIgA, radioiodinated pIgA was added to the basolateral medium of the HEC-1 cells grown on Millicell inserts. TCA-precipitable radioactivity corresponding to 54.2 ng of pIgA was detected in the apical media after a 90 min incubation at 37°C. In contrast, the apical medium of cells similarly incubated with mIgA contained TCA-precipitable radioactivity corresponding to 2.4 ng mIgA, which represents 4% of the level recovered from the filters incubated with pIgA. Following an additional hour of incubation, the levels increased to 66.8 ng of pIgA and 2.9 ng of mIgA in the apical media. Thus, specific binding occurred, followed by transepithelial transport of pIgA from the basolateral to apical medium, while the transfer of mIgA to the apical media occurred at very low levels.

To determine the time course of transport of pIgA, replicate filters with polarized monolayers of HEC-1 cells were incubated with radiolabeled mIgA or pIgA and the apical medium was harvested after 1, 2, 3, and 4 h of culture. The results presented in Fig. 8A show that much higher levels of pIgA compared with mIgA were detected in the medium from the apical compartment of the epithelial cells and that most of the IgA was transported during the first hour of incubation. Additional incubation beyond the second and third hours resulted in only a small increase of transcytosed immunoglobulin (Fig. 8A). These data indicate that the binding and transport of pIgA are rapid, occurring within the first hour

FIG 6. Expression of pIgA receptors on HEC-1 monolayers. Phase contrast micrographs are shown in panels a and c. Confluent HEC-1 cell monolayers on 24-well cluster plates were washed twice with PBS. To assay for basolateral antigen expression, unfixed cells were incubated for 10 min at 37° C with 30 mM EGTA in PBS. For comparison, duplicate wells were incubated with PBS alone. EGTA-treated or untreated monolayers were blocked with 1% BSA in PBS for 30 min on ice and treated with 95μg/ml fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human SC for 30 min at 4°C. The monolayers were washed with cold PBS to remove unbound antibody and fixed with 2% formaldehyde for 1 h at room temperature. The cells were then covered with Vectashield mounting medium and examined with a Nikon Optiphot microscope. Control wells treated with rabbit anti-CD8 immunoglobulin conjugated to FITC showed no fluorescence (not shown).

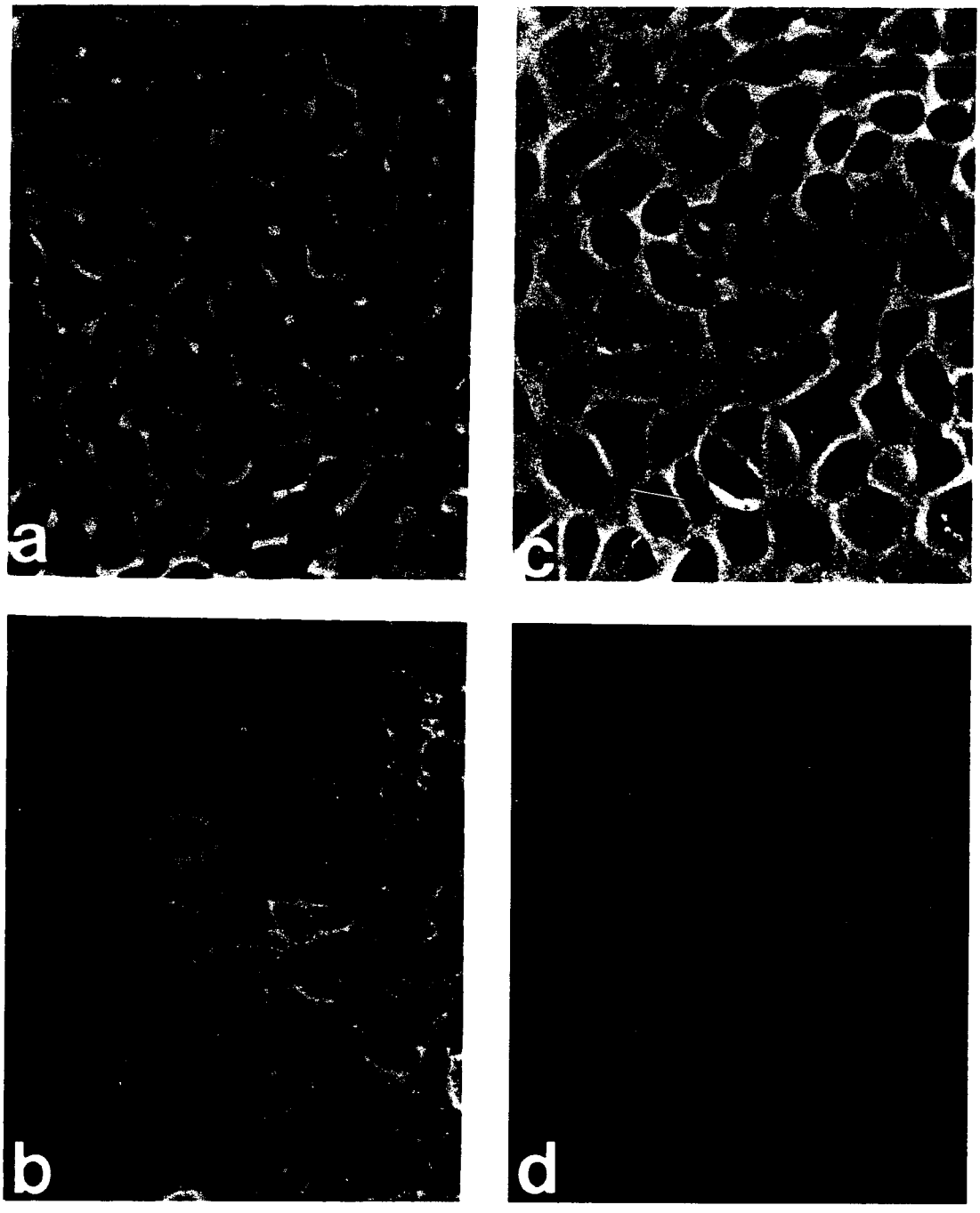
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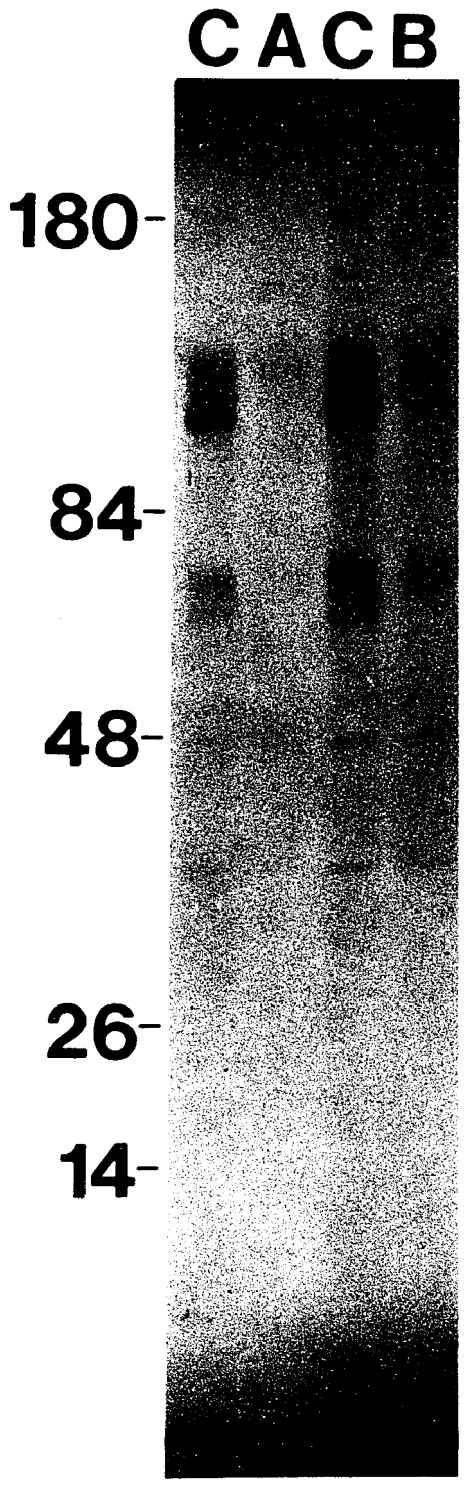
FIG 7. Basolateral expression of the pIgA receptor. HEC-1 cells were grown to confluence on permeable membrane supports and metabolically labeled for 3 h, and apical or basolateral surfaces were treated with NHS-SS-biotin as described in Materials and Methods. Cell lysates were treated with 50 μl of formalin-fixed *Staphylococcus aureus* (10% slurry) and 1 μl of normal rabbit serum for 12-16 h at 4°C. The bacteria were removed by centrifugation and the supernatants were treated with 4 μl of polyclonal rabbit antisera to human SC and 10 μl of immobilized Protein A (50% slurry) for 12-16 h at 4°C. Following four washes with lysis buffer, 60% of the sample was transferred, solubilized in 10 μl of 10% SDS, and heated at 100°C for 5 min to release the bound proteins. The eluted proteins were diluted in lysis buffer supplemented with 0.2% BSA and treated with immobilized streptavidin. The immune precipitates from cell lysates are shown in lane C; the biotinylated proteins in lysates are shown in lanes A (apical biotinylation) and B (basolateral biotinylation). Molecular weight markers are indicated on the left.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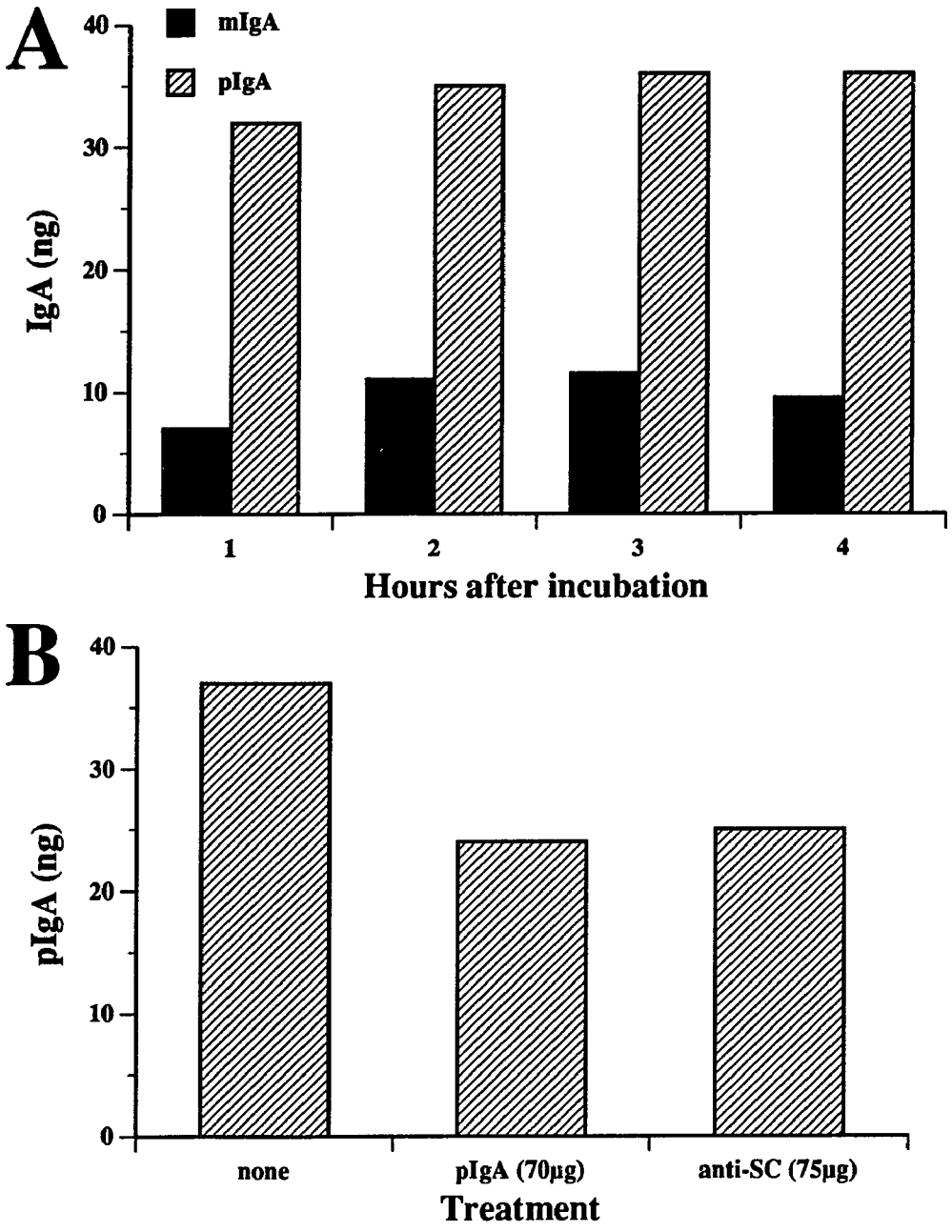

FIG. 8. Transcytosis of pIgA in HEC-1 cells grown on filter inserts. Radioiodinated mIgA and pIgA were allowed to bind at 0°C to the basolateral surface of HEC-1 monolayers. The culture inserts were incubated at 37°C and IgA transcytosis was determined at the intervals shown by measuring TCA precipitable counts in the apical medium (panel A). The results shown are averages of duplicate samples. To show that transcytosis is pIg receptor-mediated, the basolateral surface of HEC-1 monolayers were pretreated with anti-SC or cold pIgA for 1 h at 0°C. Radioiodinated pIgA was added to the basolateral compartment, the culture incubated at 37°C for 4 h, and TCA precipitable counts from the apical medium determined (panel B).

of incubation. Similar results have been reported for transfected MDCK (37) and rabbit mammary cells (53) expressing the pIg receptor.

To provide evidence that the transcytosis of pIgA was mediated by the pIg receptor, the basolateral surfaces of polarized HEC-1 monolayers were preincubated with polyclonal goat IgG anti-human SC. A 36.4% reduction in the total amount of radioiodinated pIgA transported to the apical side was observed in the filters treated with anti-SC, when compared to the untreated filters (Fig. 8B). A 32.5% reduction in the transport of radioiodinated pIgA was observed when cold pIgA was used to compete for receptor (Fig. 8B). Therefore, we conclude that polarized HEC-1 cells express pIg receptor on the basolateral membrane which is responsible for specific binding and transcytosis of pIgA.

Discussion

In this report, we describe a cell line derived from human endometrium that displays a polarized morphological phenotype, releases enveloped virus particles at distinct plasma membrane domains, vectorially secretes endogenous proteins, expresses distinct proteins on apical and basolateral surfaces, expresses the pIg receptor on basolateral surfaces, and specifically transports pIgA across the cell monolayer. These properties indicate that HEC-1 cells can be utilized to further explore the transport pathways of membrane and secretory proteins and their regulation in endometrial epithelial cells, as well as the interaction of microorganisms, including viruses, with epithelial cells of the human female genital tract.

The finding of polarized maturation of influenza virus and VSV in HEC-1 cells is similar to results obtained with other epithelial cell lines. The apical release of influenza virus, as well as basolateral maturation of VSV, have been observed in epithelial cell types originating from a variety of tissues, including kidney (48), intestine (46), mammary (51), thyroid (40), and retinal pigment cells (7). Since the polarized release of enveloped viruses reflects the site of expression of viral envelope proteins (24,49,57), the envelope proteins of these viruses are apparently recognized by similar sorting pathways in a variety of

epithelial cell types. In contrast, significant differences among cell types have been recognized for the sorting of other membrane proteins, as well as secretory proteins (for review, see ref. 47).

Polarized epithelial cells grown on permeable supports have been used extensively as model systems to study the interaction of viruses with epithelial surfaces (for review, see ref. 61). The site of expression of viral receptors defines the membrane domain at which the infectious entry process can be initiated, and a number of examples have been reported in which viral entry is restricted to a specific plasma membrane domain. Similarly, virus release is generally confined to a specific plasma membrane domain. These aspects of the cell biology of virus infection are likely to play an important role as determinants of the pathogenesis of virus infection, including the distinction between systemic and surface infections, as well as the pattern of shedding of viruses from infected hosts. Confluent monolayers of MDCK (kidney) and Caco-2 (intestinal) cells on permeable supports have also been used to study factors which are involved in bacterial adherence and invasion (16), as well as the ability of specific IgA antibodies to protect epithelial surfaces against bacterial infection (25). Thus, the finding of a polarized endometrial cell line may provide a valuable model system for similar studies with epithelial cells derived from endometrial tissues.

Similar to MDCK cells (52), different sets of endogenous proteins of HEC-1 monolayers were present on apical and basal surfaces, indicating the presence of a stringent sorting mechanism. Analogous to the protein pattern observed on the HEC-1 basolateral surfaces, multiple bands ranging from 40-150 kD were seen on MDCK basolateral surfaces (52). Two unique apical proteins (about 62 and 20 kD) were detected in HEC-1 cells. It is surprising that a more complex pattern of apical proteins was not observed using the biotinylation procedure. However, this may result at least in part from the inaccessibility of reactive amino groups to the biotinylation reagent (56).

Several studies have examined the release of secretory proteins from polarized epithelial cells. In MDCK cells, endogenously expressed proteins were observed to be

directionally secreted from either the apical or basolateral surfaces, whereas nonpolarized secretion was observed for proteins normally targeted to secretory granules when their coding sequences were introduced into MDCK cells by transfection (9,18). In contrast, all the major endogenous proteins, as well as exogenous proteins expressed from transfected genes, appear to be secreted basolaterally in hepatocytes and the Caco-2 line of intestinal epithelial cells (3,46). The directional release of secretory proteins presumably involves a specific interaction with membrane-associated components exposed in the lumen of vesicles involved in constitutive secretion. The present finding that all the detectable secretory proteins are released at basolateral surfaces of HEC-1 cells suggests that the exocytic pathway is predominantly directed to the basolateral surface in this cell type.

The pIg receptor which mediates binding and translocation of polymeric IgA and IgM across glandular or mucosal epithelia plays a vital role in mucosal immunity (34,38). Many of the functional studies of IgA transport have been restricted to animals or perfused organs due to the lack of a reliable continuous cell line that is functionally polarized *in vitro*, expresses an adequate level of the pIg receptor on the basolateral surface, and can facilitate pIgA transcytosis. To circumvent these problems, the cDNA for the rabbit pIg receptor was introduced and expressed in the polarized MDCK cell line which does not normally produce this receptor (37). This study revealed that the pIg receptor expressed in culture functions as it does *in vivo*; pIgA was bound, endocytosed, and transported across the cell monolayer. Furthermore, internalized pIgA but not IgG specific for Sendai virus was shown to neutralize intracellular virus (31). We have observed that a continuous cell line of human origin endogenously expresses the pIg receptor and binds and transports human pIgA in culture. Thus, host mucosal defenses and mechanisms of intracellular neutralization can be explored *in vitro* with the HEC-1 cell line which naturally expresses SC. Furthermore, the role of cytokines involved in the regulation of SC expression and increased binding of pIgA can be investigated and related to the potentially enhanced intracellular neutralization of viruses that infect the endometrium.

It has been reported that the level of pIg receptor expression on HEC-1 cells is enhanced in the presence of estrogen (32). These data correlate with earlier evidence that the occurrence of immunoglobulins in cervico-vaginal secretions is dependent on the hormonal status and the presence of the uterus (27,64). The availability of a human endometrial cell line sensitive to hormones and cytokines (32) should facilitate studies concerning the regulation of Ig transport in the genital tract. The presence of specific antibodies in cervico-vaginal secretions suggests that there is an induction of an immune response to foreign antigens in the female genital tract. For example, when inactivated poliovirus was used as a local immunogen, virus-specific IgA and IgG antibodies were detected in vaginal secretions and specific IgG in the uterus (42). Similarly, specific IgA and IgG antibodies to HIV-1 and -2 have been demonstrated in vaginal fluids of HIV-infected females and may play a role in antiviral immunity (4,5,43). Recently, it has also been demonstrated that oral or intratracheal immunization of female rhesus macaques with SIV resulted in the appearance of SIV-specific antibodies in vaginal secretions (30). Oral or rectal immunization of mice with cholera toxin was also observed to result in specific IgA response in vaginal secretions of mice (21). Thus, the female genital tract is an effector site for IgA secretion after the induction of common mucosal immune responses in remote mucosal tissues.

Female genital tract secretions contain antibodies which originate from two sources, 1) plasma and 2) local plasma cells (27). Transport of specific antibodies to/from the circulation to/from the female genital tract appears to play a vital role in local immunity, although additional studies are needed. The polarized HEC-1 cell line therefore provides a useful system to help elucidate the prevalence, role, and mechanisms of immunity in the genital tract.

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