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Immunology

Immunological studies of the basis for the apathogenicity of simian immunodeficiency virus from African green monkeys

(human immunodeficiency virus/animal model/pathogenesis)

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ABSTRACT **Potential reasons for the lack of pathogenicity of the simian immunodeficiency virus SIV_{agm} in its natural host, the African green monkey (AGM, *Cercopithecus aethiops*), were investigated with respect to immunological mechanisms. The functional immune response of monkeys to infection was similar (though not identical) to that of humans to infection with human immunodeficiency virus type 1 (HIV-1). In the sera of infected animals, neutralizing antibodies were found to be low or absent, and in particular there was no neutralization of the various isolates by homologous sera. There was no detectable antibody/complement cytotoxicity, though AGM sera were able to initiate antibody-dependent cellular cytolysis of infected cells in the presence of healthy effector peripheral blood lymphocytes. As in the human/HIV system, macrophages from AGMs are readily infected by SIV_{agm}. Two possibly important differences between the AGM/SIV_{agm} system and the human/HIV system are (*i*) the low immune response of the AGMs to the core protein of SIV_{agm} and (*ii*) the significantly lower inhibitory effect of SIV_{agm} proteins on the proliferation of AGM lymphocytes.**

The isolation and characterization of various members of the simian immunodeficiency virus (SIV) group from lower primates have facilitated the detailed analysis of the immune responses and diseases that result from infection by these human immunodeficiency virus (HIV)-related lentiviruses, and have allowed extensive vaccination and challenge experiments to be performed (1). It is now important to gather as much information as possible concerning each of these viruses and the manner in which they interact with the host so that comparisons at the molecular and immunological level can be made. Much of the attention in this field centers around SIV_{mac}, originally isolated from rhesus monkeys (2), although the virus (SIV_{agm}) isolated from the African green monkey (AGM) (3–5) has two properties that make it an important subject for investigation: (*i*) the AGM represents the natural host of the virus, as demonstrated by its presence in wild animals (3), and (*ii*) AGMs harboring SIV_{agm} remain healthy despite the presence of an actively replicating lentivirus.

Although it is always difficult to compare two different viruses, defining the reasons for the failure of SIV_{agm} to induce an AIDS-like disease could provide clues for the design of putative AIDS vaccines or therapeutics and several possible explanations can be postulated. The virus may be inherently apathogenic as a result of a long period of evolution within the host animal, which could manifest itself as a failure to infect and destroy the function of those cells involved in the immune response (e.g., macrophages) or as a failure of free viral proteins to interfere with the function of the immune system. Alternatively, the AGM may, for reasons unknown, be genetically resistant to the pathogenic effects of the infection. Finally, the AGM may respond to SIV_{agm} infection with a particularly vigorous immune response that is able to keep the level of infection at a tolerable level. In contrast, the failure of SIV_{agm} to induce a particular immune response might indicate an inherent lack of autoimmunity.

This is a study of SIV_{agm} carried out to seek clues as to why it causes no disease in its natural host. We report that the immune response to SIV_{agm} infection in AGMs is fundamentally similar, albeit not identical, to the known responses of humans to HIV infection and that AGM macrophages are readily infected with SIV_{agm}. Two possibly important differences are that AGM lymphocyte function did not appear to be suppressed by the presence of SIV_{agm} proteins and that AGMs appeared to be inherently low responders to SIV_{agm} core protein.

MATERIALS AND METHODS

Cells and Virus. MOLT-4 clone 8 (MOLT4/8) cells were kindly provided by M. Hayami (Kyoto University, Japan) and the SIV_{agm}-sensitive subclone MOLT4/8-79 was produced by subsequent single-cell cloning. The isolation, characterization, and purification of the SIV_{agm} strains used in these studies have been described (4).

Virus Titration and Neutralizing Antibody Assay. Heat-inactivated sera from AGMs were diluted in 2-fold steps and aliquoted into tissue-culture microtiter plates (10 μl per well, four replicates per plate, four plates). Samples (10 μl) of each of four SIV_{agm} isolates diluted to the minimum challenge dose [7 tissue-culture 50% infectious doses ($TCID_{50}$) per well, pretitrated using a precise arithmetic dilution series] were added to the relevant plates, mixed by agitation in an orbital plate-shaker, and incubated for 1 hr at 37°C. Two hundred microliters of a MOLT4/8-79 suspension (2×10^3 cells per well) was added to each well and the plates were incubated for 7 days before they were scored by microscopic examination for the presence of characteristic syncytia.

ELISA and Western Blot. Sera were tested for binding antibodies by ELISA and immunoblot with standard protocols (6, 7).

Antibody-Dependent Cellular Cytotoxicity (ADCC). For target cells, chronically infected and uninfected MOLT4/8 cells were labeled with $Na^{51}CrO_4$ (Amersham; 200 μCi per 10^6 cells; 1 μCi = 37 kBq) and thoroughly washed. Targets were

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Abbreviations: ACC, antibody/complement cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; AGM, African green monkey; HIV, human immunodeficiency virus; PBL, peripheral blood lymphocyte; PHA, phytohemagglutinin; RT, reverse transcriptase; SIV, simian immunodeficiency virus; $TCID_{50}$, tissue-culture 50% infectious dose.

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incubated in U-well microtiter plates (10^4 cells per well, 50 μl) with 50 μl of antiserum (diluted 1:100) for 20 min before addition of normal human (or occasionally AGM) effector peripheral blood lymphocytes (PBLs, 2.5×10^5 per well; 100 μl). After 4 hr of incubation at 37°C, plates were centrifuged ($200 \times g$, 5 min) and 100 μl of supernatant from each well was transferred to a tube for measurement of radioactivity in an LKB γ counter. Percent ADCC lysis was calculated as described (8).

Antibody/Complement Cytotoxicity (ACC). Assays for ACC were performed in a similar manner to the ADCC assays except that 100 μl of low-toxicity rabbit complement was used in place of the PBL effector cells and that assays were performed in flat-bottomed microtiter plates. Optimal conditions for lysis of target cells (complement dilution, etc.) were determined using CD4-specific monoclonal antibody (Clonab T4, Biotest, Dreieich, F.R.G.).

Suppression of Mitogenic Stimulation. Freshly prepared AGM or human PBLs were added to the wells of U-well microtiter plates at 10^5 per well in the presence of various concentrations of UV-inactivated virus (SIV_{agm} or HIV-1). After 3 hr at 37°C, cells were stimulated by addition of phytohemagglutinin (PHA, 10 μg/ml). After 48 hr of incubation in the absence of exogenous interleukin 2, the cultures were incubated 18 hr with [^{3}H]thymidine (1 μCi per well) before harvesting and measurement of incorporated isotope to allow calculation of the percentage inhibition of mitogenic stimulation.

Macrophage Infection Studies. AGM monocytes were prepared from peripheral blood leukocytes of seropositive and seronegative AGMs by adherence to fetal bovine serum-treated (30 min, 4°C) tissue-culture flasks (25-cm^2, $5–10 \times 10^6$ cells per flask). After 90 min at 37°C, nonadherent cells were removed and the flasks were washed twice with cold phosphate-buffered saline before addition of medium containing granulocyte/macrophage-colony-stimulating factor (Boehringer Mannheim, 100 units/ml). After 3–5 days of incubation, activated macrophages were infected by addition of 3 ml of stock SIV_{agm} suspension (5×10^5 $TCID_{50}$ per ml) for 1 hr followed by extensive washing. Medium (without interleukin 2) was changed every 5 days. Cultures were shown to be >98% monocytes by nonspecific esterase stain. Infection of macrophages was monitored by microscopic examination, by the level of reverse transcriptase (RT) in the supernatant, by cocultivation with MOLT4/8 cells, and by immunoperoxidase staining as described (4, 9).

RESULTS

AGMs Develop Little or No Neutralizing Antibody. In order to demonstrate any neutralization of SIV_{agm}, it was necessary to use a challenge dose of only 7 $TCID_{50}$ per well, the minimum found to guarantee infection of each of the 24 control wells. Table 1 shows that of the sera from infected AGMs tested, only 3 exhibited any degree of neutralization. Patterns of neutralization varied, with one monkey serum (AGM 30) neutralizing all four SIV_{agm} isolates and another (AGM 43) showing relatively strong neutralization (1:32) of one strain but not of the others. The majority of sera (total of 44) failed to neutralize any of the isolates even at a final concentration of 1:2 in the virus/serum mixture. Of particular interest is that none of the isolates were neutralized by serum from the homologous animal even though the sera were collected some months after isolation of the respective viruses.

Infected AGMs Do Not Exhibit Specific ACC. As with the human–HIV system, there are no detectable SIV_{agm}-specific complement-activating antibodies detectable in the sera of SIV_{agm}-infected AGMs (data not shown). The target cells used in the assay were fully susceptible to specific lysis because the same cells were used in the ADCC assays (see below) and the susceptibility of the MOLT4/8 cells to ACC could be shown by the high levels of lysis after binding of antibodies specific for the CD4 molecule. Although the use of rabbit complement for the experiments means that the assays did not constitute a homologous system, the use of fresh homologous complement from an uninfected AGM in the presence of sera from infected animals also failed to result in specific lysis of infected target cells (data not shown).

Table 1. Neutralization of different SIV_{agm} isolates by seropositive AGM sera

Animal	Reciprocal neutralizing titer			
	SIV_{agm3}	SIV_{agm35}	SIV_{agm37}	SIV_{agm38}
3	−*	−	−	−
27	−	−	−	−
30	8	16	8	16
33	−	−	−	−
35	−	−*	−	−
37	−	−	−*	−
38	−	−	−	−*
40	−	−	−	−
43	−	32	−	−
Z1	−	−	−	−
Z2	−	4	8	−
Z3	−	−	−	−

Titers represent the reciprocal of the highest dilution (in the virus/serum mixture) giving complete neutralization in four replicate wells. Minus sign indicates no detectable neutralization at a 1:2 dilution. Sera from 35 additional infected AGMs showed no neutralizing activity against any isolate.
*Homologous virus/serum assay.

Infected AGMs Develop ADCC-Activating Antibodies. When SIV_{agm}-infected cells were exposed to antisera from infected animals and effector PBLs, high levels of target cell lysis were observed (Fig. 1), and the patterns and levels of lysis were comparable to those seen in the human/HIV system (8). The assay used also involved the routine measurement of natural killer (NK) lysis (target cells in the presence of effector cells alone) and on no occasion was there evidence of enhanced lysis of SIV_{agm}-infected cells compared with uninfected cells.

SIV_{agm}-Infected AGMs Have Little or No gag-Specific Immune Response. AGMs naturally infected with SIV_{agm} develop a strong serological response as demonstrated by antibody binding to viral proteins in Western blot and ELISA (Figs. 2 and 3). The response is predominantly directed against both the gp140 large external and the nontruncated (10) gp45 transmembrane viral envelope glycoproteins. Thus, one striking difference from the human/HIV system is the apparent lack of antibodies specific for the core antigen p28. Normally, there is no reaction detectable by Western blot or by $p28^{gag}$ ELISA (Figs. 2 and 3*a*). One out of two AGMs experimentally infected with SIV_{agm} did develop a transient, weak anti-gag response, but it is of particular interest that primates other than AGMs (e.g., *M. nemestrina*), when infected with SIV_{agm}, do develop and maintain a strong gag-specific antibody response (Fig. 3*b*).

AGM Monocytes Can Be Infected with SIV_{agm}. The antigen processing and presentation of monocytes is an essential first step in the stimulation of a specific immune response, and there is increasing interest in the role played by HIV infection of these cells in the development of immunodeficiency (11). When purified monocytes from infected or uninfected AGMs were exposed to SIV_{agm}, RT activity in the culture supernatant developed after 10 days (Table 2). Furthermore, at that time, it was possible to observe the formation of syncytia in the monocyte culture; i.e., multinucleated giant cells formed (presumably) by the fusion of infected cells with each other or with uninfected cells. When the monocyte cultures were

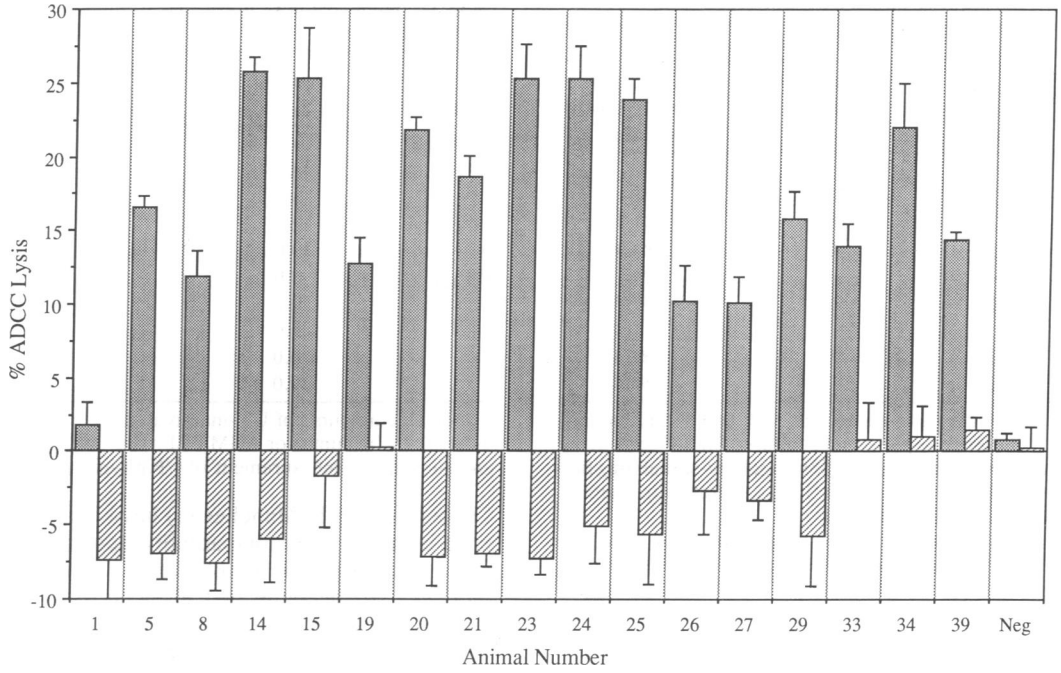

FIG. 1. ADCC directed against SIV_{agm}-infected (stippled bars) and uninfected (hatched bars) target cells by sera from SIV_{agm}-infected AGMs in the presence of human PBL effector cells. Neg, serum from an uninfected AGM.

stained by the immunoperoxidase technique, a high percentage of cells (17–43%) were shown to be infected and expressing SIV_{agm} antigen.

SIV_{agm} Protein Does Not Inhibit Cellular Proliferation of AGM Lymphocytes. It has been suggested that nonspecific inhibition of lymphocytes by HIV envelope glycoprotein (12–14) is responsible for (or contributes to) the onset of immunosuppression and hence to AIDS. When PHA-stimulated lymphocytes from seronegative AGMs were exposed to heat-inactivated, purified SIV_{agm} (for example, at 2.5 μg/ml), the degree of inhibition (3%) was significantly less than the inhibition of human lymphocyte proliferation caused by HIV-1 (52%; Table 3). This seems to be a property both of the AGM PBLs and of the virus itself, as the

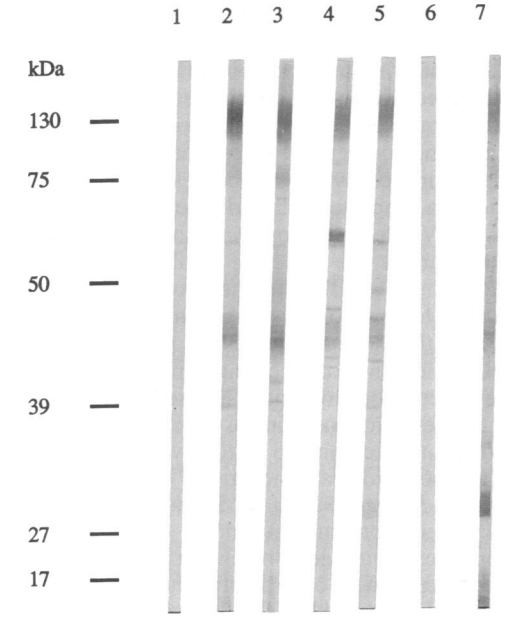

FIG. 2. Western blot analysis of sera from SIV_{agm}-infected primates with purified SIV_{agm} as antigen. Strip 1, seronegative AGM serum; strips 2–5, sera from naturally infected AGMs; strips 6 and 7, sera from a pig-tailed macaque (*Macaca nemestrina*) before infection and 3 months after experimental infection, respectively.

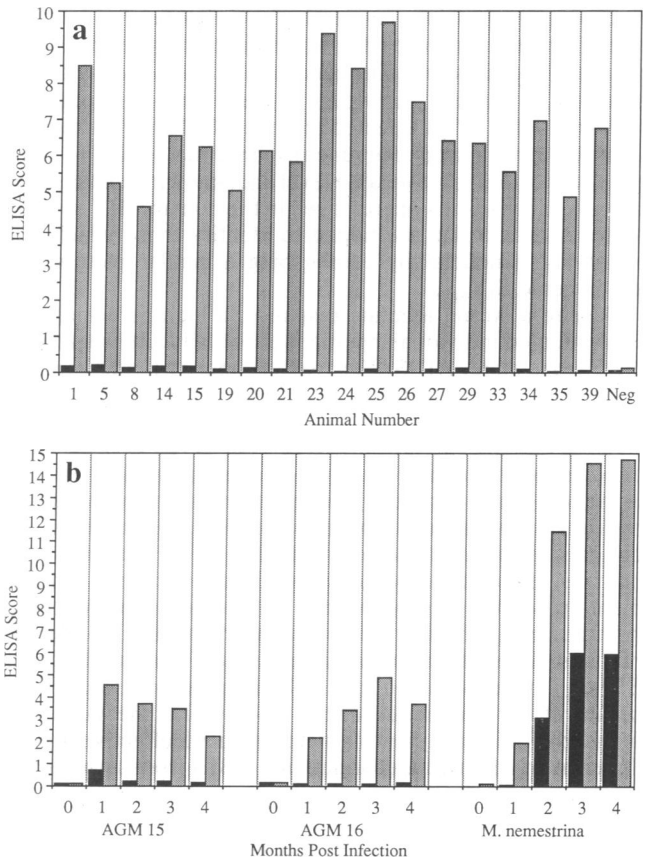

FIG. 3. ELISA reactivity of sera from SIV_{agm}-infected primates against whole virus (stippled bars) and against purified $p28^{gag}$ (black bars). (*a*) Sera from naturally infected AGMs (Neg, uninfected AGM). (*b*) Sera from artificially infected AGMs and *M. nemestrina*. Sera were titrated from 1:50 to 1:819,200 in 4-fold dilution steps. ELISA score is defined as the area under the dilution curve for each serum.

Table 2. *In vitro* infection of monocytes with SIV_{agm}

Monocyte source	IP* (10 dpi)	RT activity, cpm × 10^{-3}/ml† Monocyte culture (10 dpi)	RT activity, cpm × 10^{-3}/ml† Coculture with MOLT4/8 (12 dpi)
AGM			
46	23	5.7	ND
Z2	31	4.7	43.7
3	19	7.3	80.9
4	43	3.3	ND
8	17	2.6	37.9
Human			
Donor 1	37	9.8	89.4
Donor 2	28	5.8	50.4

Monocytes from AGMs (animals 46, Z2, 3, 4, and 8) and humans were purified by adherence to plastic and cultured for 3–5 days in the presence of human granulocyte/macrophage-colony-stimulating factor (100 units/ml) before infection with SIV_{agm} culture supernatant. dpi, Days postinfection; ND, not done.
*SIV_{agm}-specific immunoperoxidase staining; % positive cells.
†Background counts were typically in the range 0.5–1 × 10^3 cpm/ml.

inhibitory effect of HIV-1 on the monkey cells, and of SIV_{agm} on human cells, was less than the corresponding effect of HIV-1 on human lymphocytes.

DISCUSSION

There is at present no suitable model that allows a direct and precise comparison of pathogenic and apathogenic HIV strains *in vivo*. It is therefore important to accumulate as much data as possible concerning the HIV-related lentiviruses, in particular those infecting primates, to give an overall picture of each in terms of its genetics and interaction with the host, and to use this knowledge to make tentative comparisons with HIV. We suspected that the AGMs might develop a particularly vigorous immune response that is able to limit virus replication to a tolerable level and, knowing that certain immune mechanisms operate well in the human/HIV system while others do not, we hoped to identify those which are putatively beneficial by establishing a similar picture for apathogenic SIV_{agm}.

Despite a strong immune response, a high percentage of HIV-infected individuals (perhaps eventually all) progress to the disease state, AIDS. For reasons unknown, the immune system is clearly unable to maintain control of the virus. However, levels of neutralizing antibody are generally low (15, 16) and specific ACC is rarely observed (17). SIV_{agm}-infected AGMs remain healthy, and if one postulates that this is the result of a particularly vigorous immune response that restricts the virus load, these two effector mechanisms are obvious candidates. However, similar to the situation with humans, infected AGMs developed no detectable complement-activating antibodies and, furthermore, the levels of neutralizing antibodies were even lower than in HIV-infected humans. It is of particular interest that none of the four SIV_{agm} isolates was neutralized by homologous serum (i.e., serum of the individual animal from which the virus was isolated), indicating that this mechanism is ineffective *in vivo*. In terms of cell-mediated immunity, PBLs from seropositive AGMs proliferate only poorly in response to inactivated viral antigen (data not shown), similar to the situation with HIV-infected humans (18). Infected humans develop a surprisingly good cytotoxic T-lymphocyte response (19), and it is unlikely that the response in AGMs is any stronger. Preliminary experiments using as target cells autologous PHA blasts infected with recombinant vaccinia virus expressing the SIV_{agm} envelope gene (constructed by Michael Baier, Paul-Ehrlich-Institut, and Mike Mackett, Paterson Institute for Cancer Research, Manchester) have demonstrated the presence of major histocompatibility complex-restricted, envelope protein-specific cytotoxic T lymphocytes in infected monkeys (to be published elsewhere). It would therefore appear that there is no particular aspect of the humoral or cellular functional immune response that is significantly more active during SIV_{agm} infection and that could therefore account for the lack of pathogenesis.

Table 3. Suppression of mitogenic stimulation by SIV_{agm} and $HIV\text{-}1_{IIIB}$

Virus	Amount, μg	% inhibition of PHA stimulation: Human PBLs	% inhibition of PHA stimulation: AGM PBLs
$HIV\text{-}1_{IIIB}$	0.0	0	0
	2.5	52	19
	5.0	59	29
	10.0	60	26
	20.0	58	35
SIV_{agm}	0.0	0	0
	2.5	22	3
	5.0	31	6
	10.0	39	10
	20.0	40	10

Indicated amounts of UV-inactivated gradient purified virus were incubated with human or AGM PBLs for 3 hr before PHA stimulation and subsequent measurement of [^{3}H]thymidine incorporation.

It has been suggested that the decline of the immune system that predisposes the AIDS patient to opportunistic infection results from a depletion of uninfected $CD4^+$ cells by the immune system following recognition by antibody and ADCC effectors of bound HIV envelope glycoprotein (20). However, infected AGMs also develop an active ADCC system, they have a strong antibody binding response to envelope proteins, and, like HIV, the target molecule for SIV_{agm} is the simian analogue of the CD4 molecule (4). All the requirements for this mechanism are therefore met, although it is possible that there are differences between HIV and SIV_{agm} with regard to the amount of envelope glycoprotein shed *in vivo*.

One striking aspect of the serological response to SIV_{agm} is the low levels of antibody in infected AGMs to the gag protein. In Western blots, the p28 band is usually absent or extremely faint, and virtually no antibody binding to p28 in ELISA can be demonstrated. However, when the same virus is inoculated into heterologous species (*M. nemestrina* or *M. fascicularis*; ref. 21), a strong and persistent response to the gag protein is seen. The failure to develop antibodies against gag therefore appears to be a property of the primate species rather than of the virus. HIV-infected humans maintain a strong gag response throughout the infection until the development of AIDS (22). Why the AGMs, the healthy natural hosts, should be apparently low responders to gag is not clear, though one can speculate that such antibodies are potentially involved in disease induction and that the long period of coevolution has therefore selected for a low gag response. It remains to be seen whether the heterologous primate species that show a strong gag response in our colony go on to develop disease, although there is a report from another group that SIV_{agm} is in fact pathogenic in *M. nemestrina* (P. Johnson, Georgetown University, personal communication).

Macrophages, as antigen-presenting cells, play an essential role in the development of a specific immune response. These cells are readily infected by HIV-1, and it is possible that this infection *in vivo* plays a part in compromising the immune system [although the number of infected macrophages in the peripheral blood, at least, appears to be too low to fully account for this (23)]. *In vitro*, AGM macrophages are apparently as susceptible to SIV_{agm} infection as human macro-

phages are to HIV-1 infection. Naturally, these *in vitro* experiments do not necessarily reflect the situation *in vivo*, although use of the polymerase chain reaction to detect viral nucleic acid in highly purified macrophages has shown that at least some are infected *in vivo* (M. Baier, personal communication).

The inhibitory effect of inactivated SIV_{agm} on the mitogenic response of AGM lymphocytes was significantly lower than the effect of HIV-1 glycoprotein on human lymphocytes. Nonspecific inhibition of the immune system by free virus or by shed envelope protein is one possible mechanism of immunosuppression *in vivo* (12–14). These *in vitro* data suggest that such a putative suppressive mechanism *in vivo* might be much less pronounced in SIV_{agm}-infected monkeys. Comparisons using synthetic peptides of the putative immunosuppressive epitope of HIV-1 (24) and the corresponding SIV_{agm} sequence may help resolve this issue.

In summary, these studies were designed to give clues as to why SIV_{agm}-infected AGMs (the natural hosts) remain healthy. In terms of immunity, the functional immune response was shown to be similar, if not weaker, than the corresponding responses of humans to HIV. AGM cells did not appear to be resistant to infection, as SIV_{agm} readily infects AGM lymphocytes and monocytes. However, the suppressive effect of SIV_{agm} protein on cell function was less pronounced. Finally, the requirements for one possible mechanism destroying uninfected $CD4^+$ cells (antibodies to envelope glycoprotein and a functional ADCC system) were demonstrated. However, one difference that may be important was the apparent lack or low level of responsiveness of AGMs to gag protein. The immunological mechanisms studied here may or may not be involved in protection from or development of immunosuppression, and further work is needed to determine whether the putative control of SIV_{agm} replication is intracellular at the genetic level rather than at the immunological level. The apparent development of disease in heterologous primate species infected by SIV_{agm} (P. Johnson, Georgetown University, personal communication) does indicate, however, that the apathogenicity in AGM is a property of the host and not of the virus *per se*.

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