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Neutralizing Antibodies Modulate Replication of Simian Immunodeficiency Virus SIV_{mac} in Primary Macaque Macrophages

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Cultured macaque macrophages are permissive for the replication of $SIV_{mac}251$, and inoculation with virus is followed by the production of viral p27. Neutralizing macaque polyclonal and murine monoclonal antibodies preincubated with the virus prevented infection but did not prevent cytopathic virus replication when added more than 3 days after inoculation with virus. However, application of the neutralizing antibodies to macrophages 24 h after inoculation with virus resulted in sustained, low-level production of viral antigen. Cell lysates and individual macrophages from treated cultures contained less viral protein by Western blot (immunoblot) and immunocytochemistry than untreated controls. In situ hybridization and polymerase chain reaction procedures for detecting and estimating relative amounts of viral RNA and DNA showed that both viral nucleic acids failed to increase beyond the levels obtained before the addition of neutralizing antibodies. The data suggest that macrophages may need to be infected with a minimum threshold of virus particles in order to reach their full potential for virus replication and that their exposure to neutralizing antibodies prior to reaching this threshold resulted in limited virus replication.

Simian immunodeficiency virus of macaques (SIV_{mac}) is pathogenic in rhesus macaques and shares many biological properties with its human counterpart, human immunodeficiency virus (2, 3, 5, 10). $CD4^+$ T lymphocytes and monocyte-macrophages are the main cell types that support virus replication in vivo, and the dynamics of replication in these cells is thought to determine the length of the subclinical incubation period, restricted rates of virus replication being associated with prolonged infection and highly productive replication being associated with the rapid onset of disease (16). Earlier reports had shown that human immunodeficiency virus-infected patients and SIV-infected macaques that produced virus-neutralizing antibodies (NA) survived for longer periods after infection than did hosts that failed to develop these antibodies (4, 7, 9). Since viral dissemination in vivo occurs prior to the development of an NA response, the beneficial effects of the antibodies probably lie mainly in curtailing the spread of the virus from infected to uninfected cells.

In an earlier study, we used $SIV_{mac}251$, macaque macrophages, and a human T-cell line and neutralizing macaque antiserum to SIV251 to study the effects of NA on infection and virus replication in lymphocytes and macrophages (11). Our report showed that the serum prevented infection in both cell types when preincubated with virus. When added to cells that were previously infected, the serum had minimal effects on virus replication in lymphocytes. However, the addition of the serum as long as 2 days after inoculation of macrophages with virus resulted in the maintenance of continuous, low-level production of virus by the macrophages. These minimally productive infected macrophages were incapable of fusing with the lysing lymphocytes, as was commonly noted when the infected macrophages were at peak production (12). While this study showed an intrinsic difference in virus neutralization in lymphocytes and macrophages, it also suggested that NA, appearing after the establishment of virus infection in the host, could be instrumental in reducing the virus burden in the macrophage population. In this report, we show that the mechanism of this restriction was a combination of the expected inhibition of cell-to-cell spread of virus and a phenomenon of modulation of the virus life cycle in individual infected cells.

Macaque macrophages were obtained by cultivating peripheral blood mononuclear cells in RPMI containing 10% human serum and macrophage and granulocyte-macrophage colony-stimulating factors for 7 days as previously described (12). A stock of $SIV_{mac}251$ was prepared in CEM174 cells cultivated in RPMI with 10% fetal bovine serum. The antibodies used in this study were postinfectious neutralizing serum (titer, 1:10,000 in conventional neutralization assays on T cells and macrophages [12]) from a macaque (animal 4B) infected with $SIV_{mac}251$ and neutralizing murine monoclonal antibodies (KK17; titer, 1:80 in standard neutralization assays) directed against the envelope of $SIV_{mac}251$ (8). Macrophage cultures were inoculated with virus at a multiplicity of infection of about 0.05, and some of the cultures were treated 24 h later with either polyclonal or monoclonal antibodies, which were maintained in the medium. The cytopathicity of the infection was evaluated by the ability of the infected macrophages to cause lytic fusion of CEM174 cells when the latter were added to the cultures. SIV p27 levels in supernatant fluids were evaluated by a commercial

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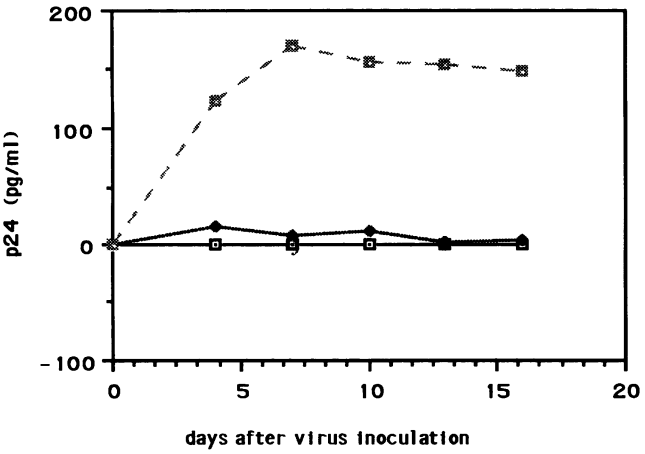

FIG. 1. Restriction of p27 production by SIV-infected macrophages treated with a 1:50 dilution of neutralizing polyclonal antiserum from macaque 4B. The addition of antibodies at the time of inoculation with virus (□) completely prevented p27 production. The addition of antibodies 24 h after inoculation did not prevent infection but severely restricted subsequent p27 production (◆). In contrast, p27 production rapidly increased in untreated infected controls (■). Each point represents the mean of measurements from three separate cultures. Standard deviation bars are not shown where the bar is smaller than the point symbol.

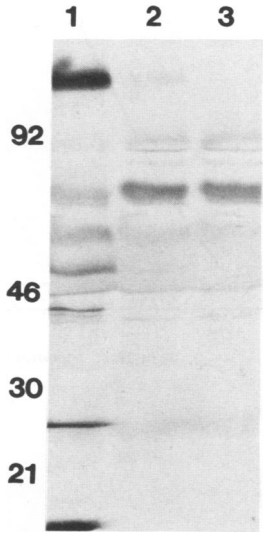

FIG. 2. Western blot of immunoprecipitated proteins from treated and control cultures. Infected, untreated macrophage cultures (lane 1) produced intense bands representing major structural proteins of the virus (gp120, p27, and p17), while cultures treated with immune serum (lane 2) produced only a faint band representing gp120. No viral proteins were detected in cultures inoculated with killed SIV (lane 3). Relative molecular weights (in thousands) are designated on the left.

immunoassay (Abbott) for the detection of p24 of human immunodeficiency virus.

The central focus of the study was the mechanism of antibody-mediated restriction of virus replication in infected macrophages treated 24 h after inoculation. The rate of production of virus core antigen was used as an indication of viral replication in macrophage cultures, and results from one representative experiment, out of five performed, are shown in Fig. 1. The inoculation of macrophages with virus resulted in increasing production of virus p27, whereas cultures inoculated with virus and immediately treated with a 1:50 dilution of neutralizing serum did not produce p27 at any time. However, cultures treated with the neutralizing serum 24 h after inoculation with virus sustained a continuous, low rate of antigen production. Following the removal of the antisera, the rate of antigen production increased to levels approximating those in untreated infected cultures, and the macrophages became fusogenic for CEM174 cells (data not shown). Postinfectious macaque serum lacking NA did not inhibit virus replication. Cultures treated 24 h after inoculation with virus with a ≤1:40 dilution of neutralizing monoclonal antibody KK17 were not fusogenic for CEM174 cells as long as the antibody remained in the culture medium; removal of KK17 from these cultures resulted in cytopathic viral replication, as evidenced by fusogenicity for CEM174 cells 7 days later. These results showed that immune serum modulated virus replication and suggested that the effect was caused by antibodies directed against a neutralizing viral epitope(s) on gp120 and not other nonneutralizing or anti-cell antibodies (15).

In order to test whether the NA could have caused sequestration of viral antigens in the cells, we immunoprecipitated proteins from lysates of treated and untreated infected cells 8 days after inoculation by using immunoglobulin from the same immune serum (macaque 4B) purified on Sepharose-protein G beads. Viral proteins were detected in Western blots (immunoblots) with immune sera from rabbits hyperimmunized with purified $SIV_{mac}251$ and peroxidase-conjugated anti-rabbit immunoglobulin (11). Figure 2 shows that minimal amounts of viral protein were present in the antibody-treated cultures, in contrast to the abundant antigen in untreated infected cultures. This experiment was repeated three times, and in one of these repeated experiments, it was not possible to detect any viral proteins in the antibody-treated cultures. The examination of ethanol-fixed preparations of treated and untreated cells for the presence of viral core antigen with monoclonal antibodies to p27 (HE3; kindly provided by Andrew Lackner, New Mexico State University) in immunocytochemical staining procedures confirmed the lower concentration of viral antigen. Antibody-treated cultures contained antigen-positive macrophages only rarely, and the staining intensity appeared to be less than that in untreated controls, in which more than 60 to 70% of the cells were strongly positive (data not shown). Thus, NA did not restrict p27 production by causing intracytoplasmic sequestration of the virus, but it did restrict the spread of the virus within the cultures and, surprisingly, appeared to prevent full expression of the virus in individual infected cells.

Next, we used in situ hybridization to examine whether NA affected the production of viral RNA, as estimated by the enumeration of autoradiographic silver grains per infected cell. Treated and untreated macrophages were harvested sequentially from individual cultures at 3- to 5-day intervals after inoculation with virus, processed for in situ hybridization as previously described (18), and hybridized to a ^{35}S-ddATP-labeled probe prepared by nick translation of full-length cloned DNA from $SIV_{mac}251$ (2). At least 200 cells from each culture were examined in order to determine the percent containing viral RNA, and the numbers of grains per cell for up to 20 cells were determined and averaged to give an estimate of the relative amount of viral RNA in a representative infected macrophage from that culture. Mac-

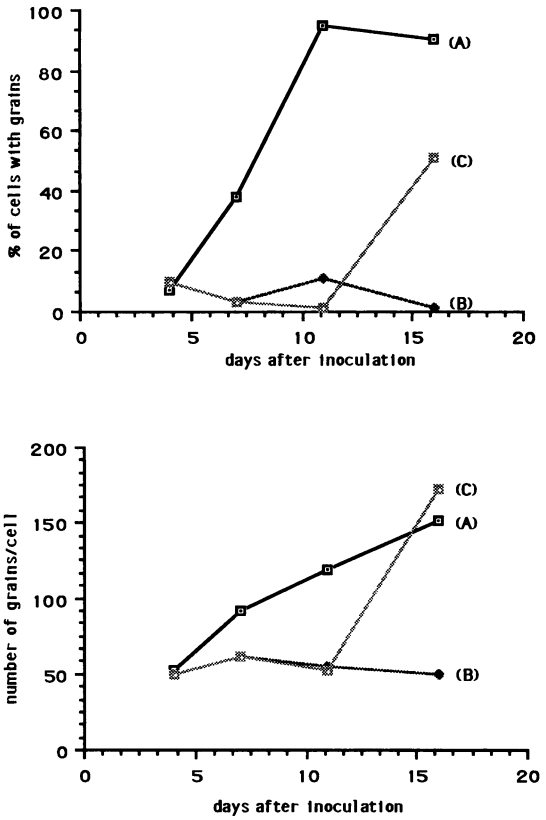

FIG. 3. In situ hybridization in macrophages inoculated with SIV 24 h before the addition of serum. Shown are the percentages of macrophages with grains and the mean number of grains per cell in cultures incubated with nonimmune serum (A) and a 1:50 dilution of immune serum (B) and those from which immune serum was removed after 7 days of incubation (C).

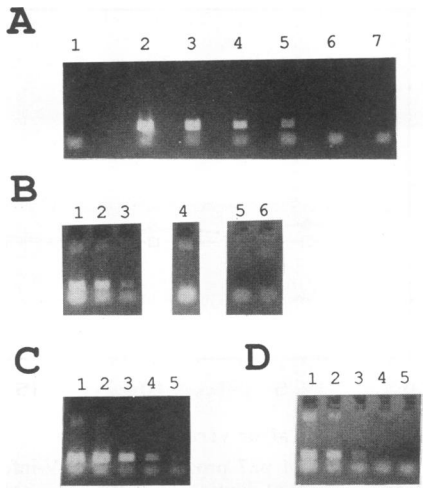

FIG. 4. Polymerase chain reaction analysis for SIV DNA. (A) Cloned template SIV DNA. Lanes: 1, negative control with no DNA; 2 to 7, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 copies, respectively, of the entire SIV genome provirus. (B) Lanes: 1 to 3, DNA harvested from cultures 24 h after inoculation with SIV; 4, a culture inoculated with heat-inactivated SIV; 5 and 6, polymerase chain reaction products obtained from the amplification of undiluted virus stock. (C and D) DNA from macrophage cultures harvested 8 days after inoculation with virus; the culture represented in panel D was treated with a 1:50 dilution of neutralizing serum 24 h postinoculation, while panel C shows amplified DNA from the untreated control. The amount of cellular DNA used for the reactions shown in panels B to D, lanes 1, was 0.2 μg; that for panels B to D, lanes 2, was 0.1 μg; that for panels B to D, lanes 3, was 0.02 μg; that for panels C and D, lanes 4, was 0.002 μg; and that for panels C and D, lanes 5, was 0.001 μg.

rophages inoculated with heat-inactivated virus did not contain detectable viral RNA, similar to uninfected controls. In four different experiments, in situ hybridization showed the same trends in the percentages of infected cells and the relative levels of viral RNA in individual cells from antibody-treated and control cultures. The results from one of these experiments are shown in Fig. 3. At the end of a 16-day experimental period, there was at least a 10-fold difference in the percentage of infected cells in treated cultures versus that of infected cells in untreated cultures and a threefold difference in the amount of viral RNA per cell (Table 1).

TABLE 1. Comparison of the mean number of autoradiographic grains per infected macrophage in antibody-treated and untreated control cultures[a]

Expt no.	No. of grains (mean ± SD)[b]	
	Antibody-treated cultures	Untreated control
1	34 ± 5 (17)	102 ± 65 (20)
2	50 ± 14 (3)	151 ± 104 (7)
3	35 (2)	133 ± 92 (20)

[a] Data are from three separate experiments and were obtained 16 days after inoculation with virus.

[b] Each number in parentheses is the number of cells examined to obtain each mean.

Thus, consistent with immunocytochemistry results, NA prevented the spread of SIV to uninfected macrophages and inhibited infected cells from reaching their full potential for production of viral RNA.

We then used the polymerase chain reaction technique to investigate whether NA treatment affected the production of viral DNA. Total cellular DNA was extracted from approximately 5×10^5 macrophages and quantitated and amplified as previously described (11), except that cellular DNA and known amounts of cloned $SIV_{mac}251$ DNA were serially diluted and used as templates for these reactions. This approach allowed a relative quantitation of the amounts of viral DNA in these cultures. Reaction products were separated by electrophoresis in a gel containing ethidium bromide and visualized with UV. Representative results from one of three experiments are shown in Fig. 4. No viral DNA was detected in the undiluted virus stock used to inoculate the cultures, but it was clearly present in macrophages 24 h after inoculation with virus. The viral DNA in the untreated cultures was estimated to increase at least 20-fold during the next 8 days (Fig. 4). In contrast, macrophages treated with antiserum 24 h after inoculation with virus and harvested on day 8 contained the same relative amount of viral DNA as cultures harvested 24 h after inoculation with virus. As expected, when NA were removed from the cultures 8 days after inoculation and DNA was harvested on day 22, the quantity of viral DNA approximated that found in untreated control cultures (data not shown).

These experiments show that NA restricted the replication of SIV in macrophage cultures when added during the early

stages of infection by preventing the spread of the virus within the culture and by modulating virus expression within individual infected cells. This restriction of SIV replication in individual macrophages may have been caused by inhibition of reinfection of the cells with progeny virus. Reinfection of cells with oncogenic retroviruses (17) and, more specifically, with lentiviruses (1, 6, 13, 14) has been shown to be important for infected cells to reach their maximum potential for virus replication and for development of cytopathic effects. Macrophages (and perhaps all lentivirus-infected cells) may therefore need to be infected with a minimum number of virus particles, either by inoculation at a high enough multiplicity of infection or by reinfection with progeny particles, in order to achieve its full potential for producing virus. It is not known how reinfection might promote virus production by an infected cell. Possibilities include increased amounts of structural proteins introduced into the cytoplasm by the reinfecting particles, repeated interactions (i.e., virus-binding events) at the cell surface, and/or an increased chance of permissive integration of viral DNA into the host genome. Whatever the mechanism, the addition of NA before the virus achieved this threshold level of infection in the macrophage prevented the full-scale replication cycle. This threshold concept of infection may be relevant in vivo, since it is possible that tissue macrophages infected with a subthreshold inoculum of virus prior to the development of an NA response by the host will remain indefinitely in the phase of slow replication subsequent to the development of an NA response.

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