Early phenotypic and functional alterations in lymphocytes from simian immunodeficiency virus infected macaques

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ABSTRACT

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Phenotypic and functional changes in lymphocytes from rhesus monkeys (*Macaca mulatta*) were
investigated during the first 6 months after infection with SIVmac 32H. Animals preimmunized with
keyhole limpet hemocyanin (KLH) were sacrificed 1, 3, 6, 12, and 24 weeks post infection. Subset
composition and function of lymphocytes from blood, spleen, lymph node and thymus were analysed.
In addition to a rapid decline in CD4/CD8 ratios, a massive reduction in CD29+CD4+ cells was
seen in the periphery. Although depletion of this subset was observed throughout the course of this
experiment, the loss of proliferative T cell responses was most pronounced very early after infection
and partially recovered after Month 3. Polyclonal cytotoxic responses were only slightly affected. In
the thymus, a gradual, but moderate loss of CD4+CD8+ immature thymocytes, and a relative in-
crease in both CD4+ and CD8+ mature subsets was observed. Infectious virus was readily recovered
from homogenates of lymph node and spleen, but not of thymus tissue. Interestingly, however, virus
was detected in thymocytes from all infected animals by cocultivation with a simian immunodefi-
ciency virus (SIV) susceptible cell line.

ABBREVIATIONS

Ab, antibody; BSA, bovine serum albumin; BSS, balanced salt solution; DN, double negative; DP,
double positive; FCS, fetal calf serum; KLH, keyhole limpet hemocyanin; mAb, monoclonal anti-
body, PBL, peripheral blood lymphocyte; PBS, phosphate buffered saline; SIV, simian immunodefi-
ciency virus; SP, single positive.

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INTRODUCTION

A key to the development of new therapeutic modalities and vaccine strategies against AIDS is a thorough understanding of its pathogenesis. Even though the later stages of the disease have been extensively studied (Miedema et al., 1990), little is known about the pathophysiological changes that occur immediately after infection (McCune, 1991). The obvious reason is that the time of infection is usually unknown and serological diagnosis only becomes possible 6–8 weeks post infection. Furthermore, the investigation of peripheral lymphatic organs such as spleen and lymph nodes is severely limited in HIV-infected humans. Effects on the thymus of infected individuals also can usually only be studied in post mortem specimens. For that reason up to now, no complete study of the immunologic changes in blood and lymphatic organs during the early stages of HIV-infected humans has been performed. The SIV-infected rhesus monkey (*Macaca mulatta*) is a suitable animal model to study such questions, because there are striking similarities between the SIV-induced disease in rhesus monkeys and the HIV-induced disease in humans (Letvin et al., 1985; Beneviste et al., 1988; Desrosier and Ringler, 1989; Desrosier, 1990). Thus, these two genetically closely related viruses (Chakrabarti et al., 1987; Franchini et al., 1987) both use the CD4 molecule as receptor (Kannagi et al., 1985; Schnittman et al., 1989) and cause an immunodeficiency syndrome in their respective hosts (Letvin et al., 1985; Baskin et al., 1988; Beneviste et al., 1988; Fultz et al., 1989). The most important difference seen so far is that SIV usually induces disease within 2 years after infection (Letvin et al., 1985; Beneviste et al., 1988; Fultz et al., 1989) while latency in HIV-infected humans is generally longer (Letvin and King, 1990). This difference greatly facilitates the study of the AIDS-like syndrome in rhesus monkeys.

In both humans (Bowen et al., 1985; Fahey et al., 1990) and macaques (Kannagi et al., 1986), a severe depletion of CD4+ cells has been described which, in human patients, correlates with the progression of the disease. In the residual CD4+ cells severe signalling defects have been found, which may extend to the CD8+ subset (Gale et al., 1990). Investigation of the CD29^{high} (Schnittman et al., 1990) and/or CD44 positive (Willerford et al., 1990) T cell subpopulations of 'memory-cells' showed functional defects in both species and a greater viral burden as compared with CD29^{low} T helper cells (Gallatin et al., 1989; Schnittman et al., 1990; Willerford et al., 1990). In monkeys, it was additionally shown that these 'memory-cells' were selectively lost during the course of infection (Gallatin et al., 1989; Murphey-Corb et al., 1989; Willerford et al., 1990). Because of these and other striking similarities between the disease induced by HIV in humans and by SIV in macaques, we have undertaken the present study of the rhesus monkey model to analyse the

early phase of infection and the events taking place in organs usually not accessible in the humans.

MATERIALS AND METHODS

Animals

The rhesus macaques (*Macaca mulatta*) used in this study were of Chinese origin and kept at the German Primate Center, Göttingen as previously described (Stahl-Hennig et al., 1990). At the time of infection the monkeys were between 3 and 3.3 years of age (Table 1). All monkeys were seronegative for type-D-virus, STLV-1 (simian T-cell lymphotropic virus-1) and SIV. At Day 39 before infection they were immunized intramuscularly with 1 mg keyhole limpet hemocyanin (KLH) in alum with 10^9 killed pertussis-organisms. Five animals were inoculated intravenously with 1 ml of cell free stock, virus of SIVmac 251/32H (Cranage et al., 1990) containing 100 MID_{50} (monkey infectious doses infecting 50% of recipients). One additional animal served as a negative control. At Weeks 1, 3, 6, 12 and 24 after infection, individual animals were sacrificed and lymphocytes from blood, spleen, thymus and lymph nodes were isolated. The negative control was sacrificed 1 week before the end of the experiment.

TABLE 1

Date of birth, duration of infection, virus isolation, seroconversion and clinical symptoms in experimentally SIVmac infected rhesus monkeys

Animal no.	Date of birth	Sacrificed at w.p.i.	Isolation of virus w.p.i.	Seroconversion w.p.i.	Clinical symptoms
5027	15.3.87	23 (control)[1]	–	–	–
5130	16.7.87	1	1	–	–
5127	03.7.87	3	>2	3	lympha-denopathy
5016	24.3.87	6	>2	4	persisting lympha-denopathy (p.l.)
5017	07.4.87	12	>2	4	p.l.
5029	07.3.87	24	>2	4	p.l.

[1]Sacrificed at 23 weeks after experimental infection of the other animals.

Isolation and detection of virus

Fragments of freshly prepared organs were homogenized in RPMI 1640 supplemented with antibiotics and centrifuged for 30 min at 3000 r.p.m. The cell free supernatants were added to C8166 indicator cells (Salahuddin et al., 1983). Peripheral blood lymphocytes (PBLs) were prepared as described (Stahl-Hennig et al., 1990) and co-cultivated with C8166 cells after 3 days of stimulation with phytohemagglutinin. The cultures were maintained over a period of 5 weeks and regularly inspected for the appearance of giant cells and syncytia formation. Virus replication in these cultures was confirmed by a reverse transcriptase assay (Stahl-Hennig et al., 1990). To investigate virus production in cultured cells from spleen and thymus, cell suspensions were prepared after careful removal of adhering lymphatic tissue. 5×10^5 thymocytes or spleen cells were cocultured with 1×10^4 C8166-cells in 1.5 ml RPMI 1640 (supplemented with L-glutamine (2 mM), penicillin, streptomycin, and 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany)) containing 100 U human rIL-2 ml^{-1}. The cytopathic effect on the C8166 cells was determined by microscopic inspection. After 48 h of co-cultivation the supernatants were monitored in triplicates for viral antigen by an anti simian p27 ELISA (Coulter El., Hialeah, FL) according to the manufacturer's instructions.

Cytofluorometry

Cells were surface labelled by incubating 2×10^5 cells for 30 min at 4°C in 100 μl phosphate buffered saline (PBS), pH 7.2, containing 0.2% bovine serum albumin (BSA), 0.02% sodium azide and the respective antibodies. The cells were then washed in the same buffer. Indirect labelling was performed by adding PE-conjugated rat anti-mouse kappa antibody (Becton-Dickinson, Heidelberg, Germany). After another washing cycle and incubation with mouse IgG (Sigma, Deisenhofen, Germany, 10 μg ml^{-1}) for 15 min, to block free binding sites of the mouse kappa reagent, cells were stained with a FITC-conjugated antibody for 15 min. The cells were washed, fixed with 3.5% formaldehyde to inactivate SIV and analyzed in a FACScan flow cytometer (Becton-Dickinson, Heidelberg, Germany). Anti human monoclonal antibodies (mAb), which crossreact with macaque lymphocytes, were obtained from the following sources: Coulter Immunology (Hialeah, FL): CD29 (4B4, 1:200), P. Rieber (München, Germany): CD4 (MT310), CD8 (MT122). The anti rhesus CD3 mAb (FN 18) was obtained from Primate Center TNO, Rijswijk, Netherlands. The latter antibody was purified and directly FITC-conjugated (Janossy et al., 1987). All antibodies were used at saturating concentrations. Appropriate negative controls were used to set the cut off points in dot plots and to calculate the fraction of positive cells.

Cytotoxicity assay

PBL and lymphocytes prepared from spleen were cultured at 2×10^6 ml^{-1} RPMI in the presence of ConA (2.5 μg ml^{-1}) with or without 100 U human rIL-2. After 72 h alpha-methyl-mannoside was added to a final concentration of 50 mM. After 1 h, cells were washed three times and their cytotoxic activity was assessed in an antibody redirected 5 h ^{51}Cr release assay using R73 cells as targets. These murine hybridoma cells produce an anti rat alpha/beta TCR antibody (Ab) (Hünig et al., 1989) crossreacting with macaque alpha/beta TCR. Target cells were labelled for 1 h at 37°C with Na$_2$[^{51}Cr]O$_4$ (DuPont, Dreieich, Germany). ^{51}Cr labelled R73 cells (5×10^3) were incubated in triplicate with 5×10^4 effector cells for 5 h.

TABLE 2

Reisolation of SIVmac from different organs of rhesus monkeys sacrificed at times indicated. Tissue homogenates were tested for the presence of infectious virus except for PBL, which were assayed after coculture with C8166 cells. Both tests were carried out for spleen and thymus

	Animal number					
	5130	5127	5016	5017	5029	5027
Sacrifice weeks p. infection	1	3	6	12	24	control
Cervical lymph nodes	+	n.d.	+	+	+	–
Mesenterial lymph nodes	+	+	+	+	+	–
Inguinal lymph nodes	+	+	+	+	+	–
Blood lymphocytes	+	+	+	+	+	–
Spleen						
homogenized	+	+	+	+	+	–
co-culture	+ / > 1.6	+ / > 1.6	+ / > 1.6	+ / > 1.6	+ / > 1.6	– / 0.12
Thymus						
homogenized	–	–	–	–	–	–
co-culture	+ / > 1.6	+ / > 1.6	+ / > 1.6	+ / > 1.6	+ / > 1.6	– / 0.12
parotis	–	–	–	–	–	–
gonades	–	–	–	–	–	–
bone marrow	–	–	–	–	–	–

+ Refers to an observation to cytopathic effects and/or positive RT assay, the numbers are OD units determined in the p27 ELISA. Neg. co.: 0.12 OD units, pos. co.: 1.6 OD units.
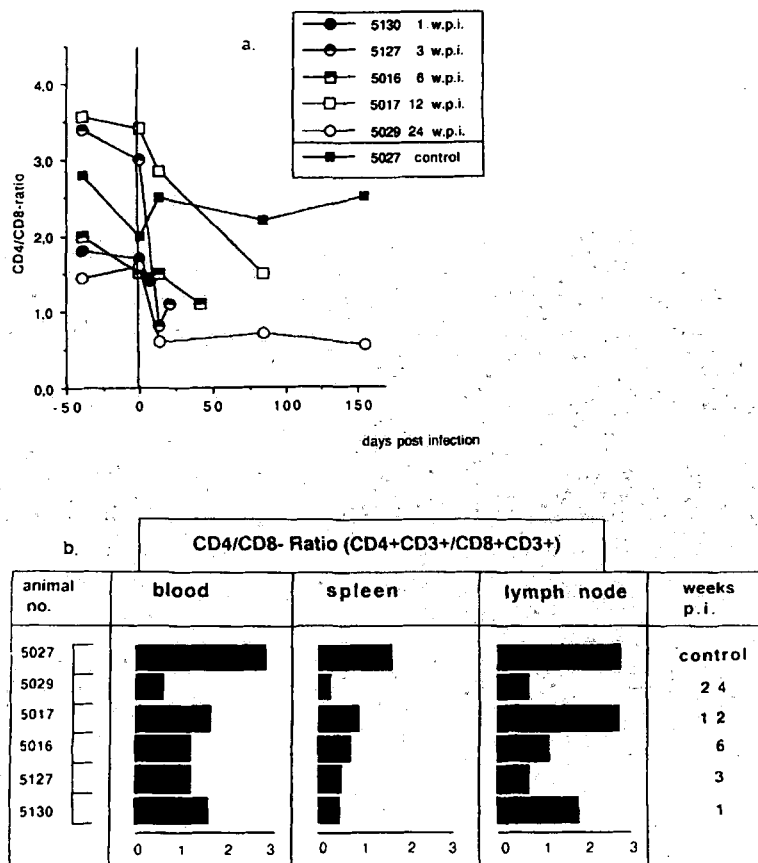
Fig. 1. (a) CD4/CD8-ratios in blood of macaques experimentally infected with SIVmac and of one control animal over time. PBL were isolated and phenotyped as described in 'Materials and Methods'. (b) CD4/CD8-ratios in lymphocytes from blood, spleen and mesenteric lymph nodes of experimentally infected rhesus monkeys sacrificed at various times after infection. Percentages of CD4+CD3+ and CD8+CD3+ cells of CD3+ cells are given.

Proliferation assay

Antibody mediated proliferation

Flat-bottom microtiter plates (Costar, Cambridge, MA) were coated over night at room temperature with rabbit anti-mouse IgG (40 μg ml^{-1} (Dako, Hamburg, Germany)) in coating buffer (0.05M Na-Carbonate, pH 9.5). After washing three times with BSS (balanced salt solution), plates were incubated for 4 h at 4°C with 1 μg ml^{-1} anti-CD3 mAb (FN18, Nooj et al., 1986). After extensive washing with BSS, 2×10^5 cells in 0.2 ml RPMI 1640 supplemented as described above, were added.

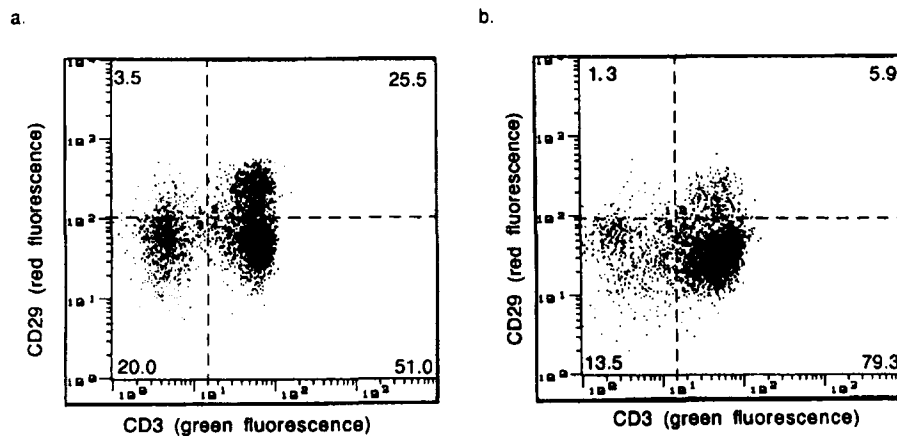a.   b.

Fig. 2. Surface expression of CD29 (red fluorescence) vs. CD3 (green fluorescence) in lymphocytes isolated from the peripheral blood of (a) an uninfected animal; (b) an animal 12 weeks after infection with SIVmac.

Antigen induced proliferation

Lymphocytes (2×10^5) were placed in culture medium in U-bottom microtiter plates (Costar, Cambridge, MA) containing 100 μg ml^{-1} KLH. The cells were cultured at 37°C for 72 h, with or without human rIL-2 (100 U rIL-2 ml^{-1}) in a final volume of 200 μl. Each well was pulsed for 10 h with 25 μCi of [^{3}H]dThd (specific activity 2Ci mM^{-1}), harvested onto glass-fiber filter strips and the radioactivity incorporated into DNA was measured in a scintillation counter. The results are expressed as median c.p.m. of triplicate cultures.

RESULTS

Seroconversion and reisolation of virus from SIVmac 32H infected animals

A group of five age matched chinese rhesus monkeys was inoculated intravenously with cell free SIVmac 32H. Animals were sacrificed at 1, 3, 6, 12 and 24 weeks post infection. The uninfected control animal from the same cohort was sacrificed 1 week before the end of the experiment. Seroconversion was observed in all five infected animals 3–4 weeks following infection with SIV (Table 1). At the same time lymphadenopathy was diagnosed, which was less severe than in later stages. The animals showed no signs of opportunistic infections or other overt disease at the time of sacrifice (i.e. up to 6
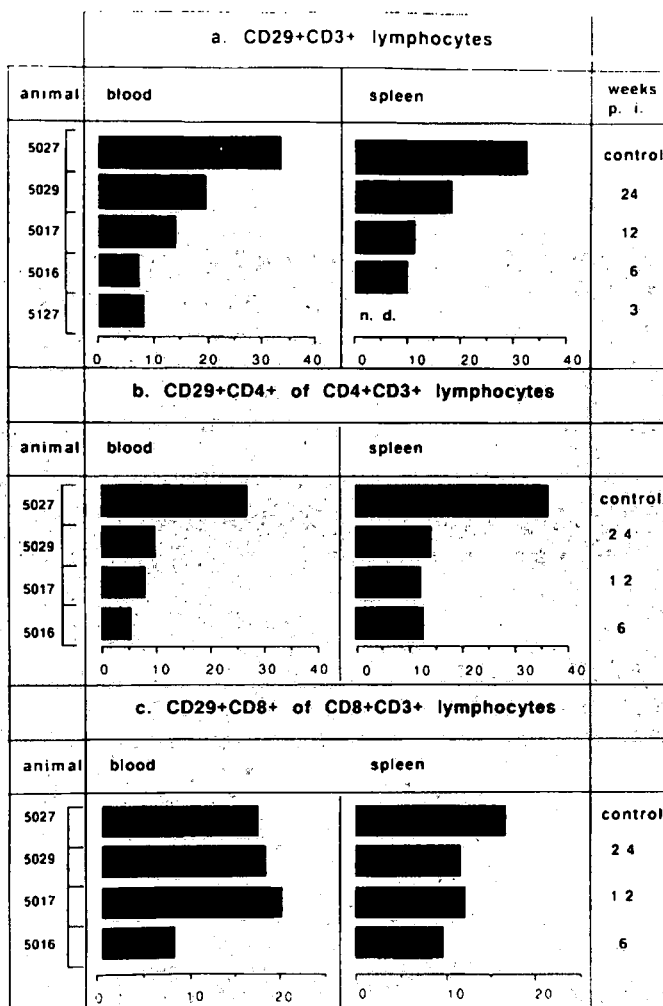
Fig. 3. Representation of CD29+ cells among T cell subsets of SIV infected animals. Lymphocytes were isolated from blood and spleen of the control and experimentally infected rhesus monkeys sacrificed at different times. (a) Percentage of CD29+CD3+ cells as percentage of total CD3+ cells. (b) Percentage of CD29+CD4+ lymphocytes of CD4+CD3+ cells. (c) Percentage of CD29+CD8+ lymphocytes of CD8+CD3+ cells.

months post infection). The virus was successfully isolated from lymph nodes, spleen and peripheral blood lymphocytes from all experimental animals (Table 2). The isolation of the virus from thymocytes will be discussed later.
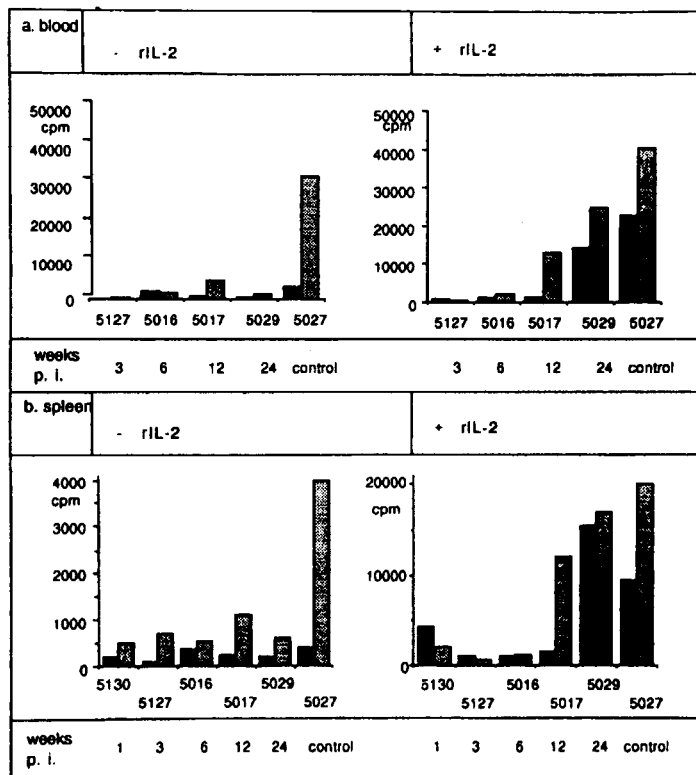
Fig. 4. Proliferation of T cells induced by KLH. Lymphocytes from (a) blood; (b) spleen of experimentally infected animals and the control were stimulated with KLH (50 μg ml^{-1}) with and without exogenous rIL-2 (100 U ml^{-1}) (bright bars). Background of cultures without KLH is shown as dark bars.

Phenotypic analysis of distinct T cell subsets in blood, spleen and mesenteric lymph nodes

Figure 1 (a) shows CD4/CD8 ratios in peripheral blood before infection and at various time points until sacrifice of the individual animals. While the control animal maintained a constant CD4/CD8 ratio of about 2.5, the ratios declined in all infected animals, though to a varying degree and with different kinetics. By 2 weeks after infection the ratio had fallen below one: two out of the five infected animals. The reduction of CD4/CD8 ratios in spleen and lymph nodes was variable between the animals sacrificed at different time points (Fig. 1 (b)). The most drastic effect was observed in the animal sacrificed 3 weeks post infection. Evaluation of the CD29+ T-cell subset, which
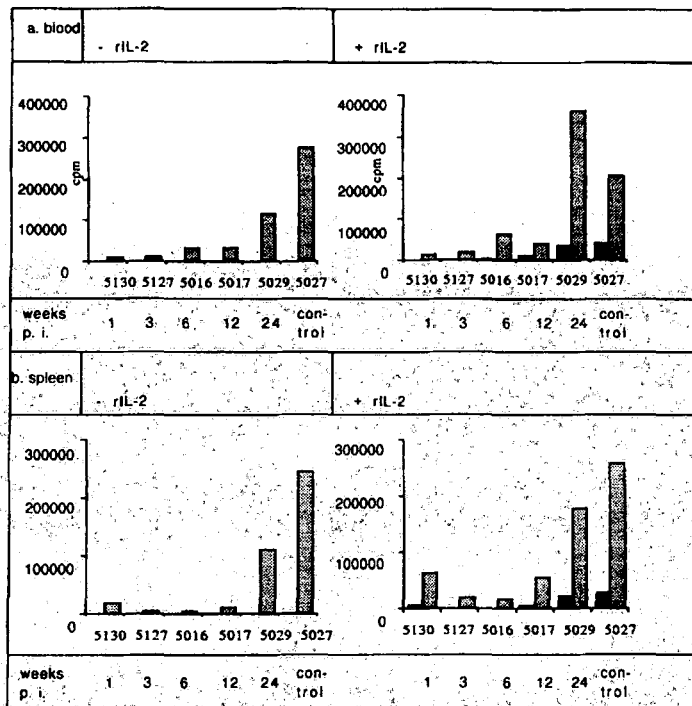
Fig. 5. Proliferation of T cells induced by immobilized anti-CD3 mAb. Lymphocytes from (a) blood; (b) spleen of experimentally infected animals and the control were stimulated with immobilized anti-CD3 mAb with and without exogenous rIL-2 (100 U ml^{-1}) (bright bars). Background of cultures without anti-CD3 mAb is shown as dark bars.

has been correlated with memory T-cells, at 3 weeks post infection revealed a nearly complete disappearance of CD3 + lymphocytes expressing this marker at a high level from the blood (Fig. 2).

Figure 3 summarizes the results obtained for all animals. There was a significant decrease of CD29 + cells in both the CD4 + and CD8 + subset of the animal sacrificed 6 weeks after infection. At later times, higher percentages of CD29 + CD8 + lymphocytes were seen again, while the CD29 + CD4 + cells were always low. Similar results were obtained with splenic lymphocytes though the depletion was less pronounced.

T cell proliferative response to KLH

As a functional readout for memory T cells the proliferative response to the recall antigen KLH was studied. In Fig. 4 it is shown that T cells isolated from blood (Fig. 4(a)) or spleen (Fig. 4(b)) of SIV infected animals had a decreased response to KLH compared with lymphocytes from the control ani-
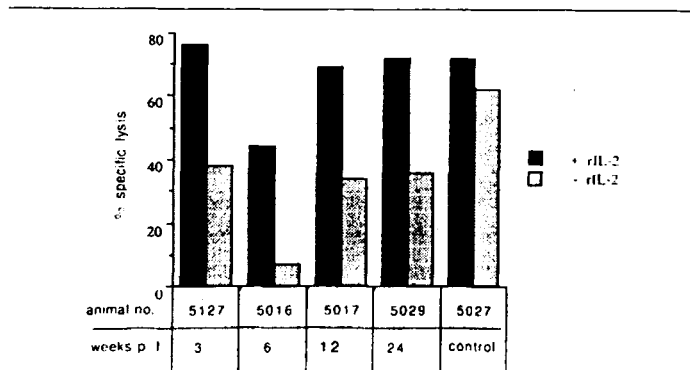
a. blood
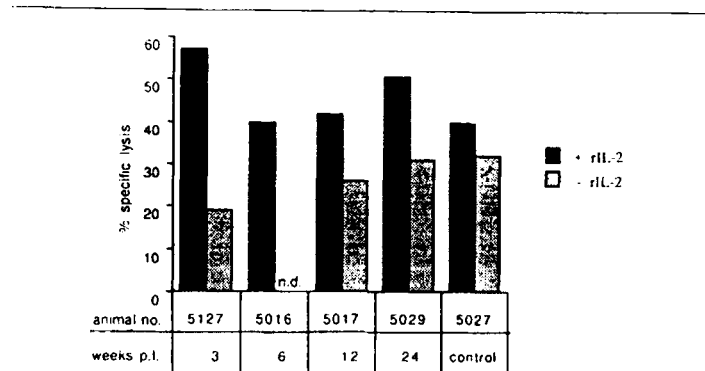
b. spleen

Fig. 6. Antibody redirected cytotoxicity of polyclonally activated lymphocytes isolated from (a) blood; (b) spleen of experimentally infected animals and the control. Cells were stimulated with ConA (5 μg ml⁻¹) alone (dark bars) or with addition of exogenous rIL-2 (bright bars) for 3 days. Effector cells were incubated with ^{51}Cr-labeled R73 target-cells for 5 h at different E:T ratios only one of which (30:1) is shown.

mal. Addition of 100 U rIL-2 partially reconstituted this defective response when cells from the animal sacrificed 12 weeks post infection were analysed. No effect of rIL-2 was seen however with cells from animals 6 weeks or earlier post infection.

T cell proliferative response to immobilized anti-CD3 antibody

To investigate TCR mediated T cell responses at a polyclonal level, lymphocytes from blood and spleen were stimulated by crosslinked anti-CD3 mAb (FN18). Figure 5 indicates that the infected animals responded poorly compared with the control animal. Again this defect was partially overcome by

TABLE 3

Distribution of T cell subpopulations in the thymus at early times post infection with SIVmac. Percentages of subsets calculated from one and two colour flow cytometric analysis performed as described in 'Materials and Methods'

Animal no.	w.p.i.	CD4− CD8−	CD4+ CD8+	CD3 low	CD3 high	CD8+ CD3 high	CD4− CD8+	CD4+ CD3 high	CD4+ CD8−
5130	1	nd	nd	48	12	4	nd	10	nd
5127	3	nd	nd	59	9	4	nd	7	nd
5016	6	6	78	52	8	3	8	6	8
5017	12	8	69	42	16	10	10	10	12
5029	24	11	62	41	23	11	10	11	17
5027	control	3	82	57	10	5	5	6	9

the addition of rIL-2 (Fig. 5). However, similar to the KLH induced response, no effect of rIL-2 was seen with cells taken at the earlier time points.

Cytotoxic activity of polyclonally activated lymphocytes

The capacity to generate cytotoxic alpha/beta T lymphocytes was investigated in vitro. T lymphocytes from blood (Fig. 6(a)) and spleen (Fig. 6(b)) were polyclonally activated by ConA for 3 days and CTL activity was determined by measuring lysis of anti-alpha/beta TCR mAb producing hybridoma cells. In the absence of rIL-2, cytotoxic activity of the cells from infected animals was slightly diminished compared with cells obtained from the control animal. Addition of rIL-2 during polyclonal activation, raised the cytotoxic capacity of cells from infected animals to the level found in the control.

Thymus

In contrast to the other lymphoid organs investigated, the infectious virus could not be isolated from the thymus homogenates. However, after co-culture of thymocytes with rIL-2 and C8166 cells, productive infection of the indicator cells was observed and assessed by cytopathic effects and a p27 ELISA (Table 2) as described in 'Materials and Methods'.

The distribution of the 'double negative' (DN), 'double positive' (DP) and 'single positive' (SP) cells in the thymus as defined by their coreceptors CD4 and CD8 is shown in Table 3. Concomitantly with a slight decrease of DP lymphocytes in thymocytes from infected animals sacrificed 6 weeks or more post infection a relative increase in SP thymocytes was observed. In contrast to peripheral lymphoid organs there was no selective loss of CD4 SP cells. The decrease in DP cells was also reflected in a reduction of CD3^{low}

(immature) and a relative increase in CD3^{high} (mature) thymocytes. Morphologic analysis also showed a cortical depletion which indicates a diminution of immature thymocytes (Müller et al., 1992).

DISCUSSION

The present study was undertaken to determine quantitative changes of lymphocyte subsets and functional alterations in the immune response during the early phase of SIVmac 32H infection in rhesus monkeys. In addition to pathophysiological alterations in peripheral blood lymphocytes this animal model also allowed us to investigate lymphocytes in spleen, lymph nodes and thymus.

The number of CD4+ cells and the CD4/CD8-ratio, in peripheral blood lymphocytes, are well accepted immunological parameters to assess the course of HIV- (Fahey et al., 1984) or SIV- (Letvin and King, 1990) induced disease. This was confirmed in the present study. A clear reduction in the representation of CD4+ lymphocytes in blood and lymphatic organs was observed very early after infection, although it was not manifest to the same extent in all animals. In addition we found a very early (at 3 weeks post infection, the first time point at which this marker was examined) and strong diminution of CD29+ T-lymphocytes in the blood. Both CD29+CD4+ and CD29+CD8+ cells showed an initial decline (Fig. 3). However, while the frequency of the CD29+CD4+ lymphocytes was low at all time points investigated (6, 12 and 24 weeks post infection), the CD29+CD8+ lymphocytes were found to be normally represented in animals sacrificed at 12 or 24 weeks post infection. The depletion of CD29+CD4+ cells is in agreement with results reported by Schnittman et al. (1990) for HIV-1 positive humans and Willerford et al. (1990) for SIVmac rhesus monkeys. Schnittman et al. (1990) suggested that the decline of CD29+ cells in HIV positive individuals was due to the preferential replication of HIV in these cells. A very early loss of CD29+CD4+ cells from the blood of SIVsm infected macaques has also been noted by Murphey-Corb et al. (1989). The low level of CD29+CD8+ cells in blood and spleen of the animal sacrificed 6 weeks post infection was not seen in the other monkeys at later times. While it cannot be excluded that the low frequency of CD29+CD8+ lymphocytes in one animal could reflect individual variations, it may, on the other hand, be a very early effect of SIV infection. Thus, immunoregulatory mechanisms that are part of the antiviral response, impairment of CD4+ T cell function, or a direct effect of the virus could all modulate homing behaviour and marker expression of the various subsets, including the CD29+CD8+ subset.

Since it has been shown (Morimoto et al., 1985; Merkenschlager et al., 1988; Akbar et al., 1991) that cells responding to soluble recall antigens in vitro express high levels of CD29, it was not surprising that the proliferative

response to KLH was rapidly lost in spleen and blood lymphocytes after infection. The response to immobilized anti-CD3 antibodies was also impaired (Fig. 5). The fact that the ability to proliferate seems to be more strongly reduced than the frequency of CD4+ cells, suggests that the residual cells may exhibit signalling defects at a very early stage after infection. Whether this is directly related to the tropism of the virus or its envelope product for CD4+ cells (Kannagi et al., 1985; Schnittman et al., 1989) or indirectly caused by immunosuppressive effects of acute virus infections remains open. In this context it is worth noting that although CD4/CD8 ratios remain low or even decline further, the response to KLH and crosslinked CD3 antibodies tends to partially recover after 12 weeks of infection.

In contrast to the impaired proliferative responses, generation of cytotoxic alpha/beta-positive T cells after polyclonal stimulation of splenic lymphocytes with ConA was only slightly affected (Fig. 6).

Besides the well documented effects of HIV or SIV infection on the peripheral immune system, interference of virus infection with T cell development seems possible. Infection of thymocytes with immunodeficiency viruses could contribute to the peripheral decline in immune function in two ways: reduction of T cell output (in young individuals) and infection of maturing lymphocytes, including those that will aquire the CD4 − CD8 + phenotype, during transit through the CD4+CD8+ compartment.

The finding that HIV can infect human thymocytes in vitro (DeRossi et al., 1990; Schnittman et al., 1990) is compatible with such mechanisms. Although in our present study no infectious virus was found in thymus homogenates (which is in contrast to homogenates from peripheral lymph nodes) co-culture of thymocytes from infected animals with a susceptible cell line demonstrated that the virus could be recovered quite readily (Table 2), indicating that the thymus is in fact a target for the virus in vivo. This has recently also been described by Baskin et al. (1991). However, the form (dormant or replicating) in which the virus is present and which of the various T cell subpopulations are infected in vivo remains to be determined. Interestingly, the ratio of the mature CD4 SP and CD8 SP thymocyte subsets was not markedly affected by virus infection, and the fraction of double positive thymocytes was only slightly reduced. Whether this reduction in DP cells (Table 3), which later, during infection, may lead to the pathomorphological changes described as cortical atrophy (Baskin et al., 1991; Müller et al., 1992) is due to virus infection per se or to indirect effects, is difficult to assess in vivo, but could possibly be addressed using organ cultures. To better understand the role of the thymus in the pathogenesis of AIDS, particularly the effects of thymus infection on the course of disease in children and juveniles, further investigations will be aimed at determining the stage and the extent of infection in the thymocyte subpopulations.

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