Characterization of the Cutaneous Exanthem in Macaques Infected with a Nef Gene Variant of SIVmac239

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The molecularly cloned viruses known as SIVmac239/R17Y and SIVmac239/YEnef cause extensive lymphocyte activation and induce an acute disease syndrome in macaque monkeys. One manifestation of this syndrome is a severe diffuse cutaneous maculopapular exanthem that is similar to the exanthem associated with HIV-1 infection. To examine the pathogenesis of this exanthem, biopsies obtained throughout the course of clinically evident rash were examined for the presence of virus by *in situ* hybridization and immunohistochemistry, and the cellular infiltrate was characterized with respect to cellular immunophenotype and chemokine receptor expression. The onset of rash was associated with abundant simian immunodeficiency virus nucleic acid and protein within perivascular dermal infiltrates and occasionally within intraepithelial cells. Analysis of cellular infiltrates showed that biopsies, obtained on the day of rash onset, were composed of equal numbers of CD4+ and CD8+ lymphocytes and abundant αEβ7 positive cells surrounding vessels with upregulated endothelial E-selectin. Moreover, by examining virus expression in sequential skin biopsies from the same animal, the clearance of virus and the resolution of rash were associated with an increase in the percentage of cells expressing CD8, the chemokine receptor CXCR3, and GMP-17, a marker of cytotoxic granules. These results suggest that activated cytotoxic T cells are trafficking to sites of inflammation in the skin and directly or indirectly affect levels of viral replication at these sites. *Key words: CXCR3/lymphocyte/rash/skin. J Invest Dermatol 110:894–901, 1998*

The acute exanthem associated with primary human immunodeficiency virus (HIV) infection is one of many clinical symptoms associated with the mononucleosis–like illness that arises prior to seroconversion (Cooper *et al*, 1985). Other common manifestations are fever, malaise, myalgia, headache, pharyngitis, and lymphadenopathy (Cooper *et al*, 1985; Lindskov *et al*, 1986). HIV exanthem is the most common of these clinical syndromes, occurring in up to 70% of patients during primary HIV-1 infection (Clark *et al*, 1991; Kinloch-de Loes *et al*, 1993; Fiscus *et al*, 1995; Lapins *et al*, 1996). This nonpruritic roseola–like rash consists of round to oval erythematous macules and papules localized mostly to the trunk, with the face and extremities as the second and third most common sites affected (Berger *et al*, 1988; McMillan *et al*, 1989; Kinloch-de Loes *et al*, 1993; Alessi and Cusini, 1995; Duvic, 1995; Goldman *et al*, 1995; Lapins *et al*, 1996; Martinez-Escribano *et al*, 1996). Histopathologic examination of skin lesions has shown a perivascular lymphohistiocytic infiltrate of the superficial dermis with epidermal spongiosis and parakeratosis (McMillan *et al*, 1989; Balslev *et al*, 1990; Hulsebosch *et al*, 1990; Goldman *et al*, 1995; Lapins *et al*, 1996; Martinez-Escribano *et al*, 1996). These histopathologic and immunohistologic features of HIV exanthem are

not unique to HIV infection and resemble those of other viral infections (McMillan *et al*, 1989; Balslev *et al*, 1990; Duvic, 1995). Moreover, although this acute syndrome is associated with high levels of viremia (Clark *et al*, 1991; Daar *et al*, 1991), HIV is not uniformly observed in skin lesions (McMillan *et al*, 1989; Duvic, 1995; Lapins *et al*, 1996). Hence, the pathogenesis of this exanthem remains an enigma.

To investigate the pathogenesis of the HIV exanthem, investigators have used the simian immunodeficiency virus (SIV)-infected macaque monkey model of AIDS (Ringler *et al*, 1986, 1987; Baskin *et al*, 1988). SIV is a lentivirus that has similar genomic organization and gene sequences to HIV (Desrosiers, 1990) and induces both acute and AIDS–like disease syndromes in macaque monkeys (Letvin *et al*, 1985; Reimann *et al*, 1994). Within a few weeks of infecting macaque monkeys with most strains of SIV, an erythematous maculopapular eruption can occur, which is histologically identical to that described in primary HIV-1 infection (Ringler *et al*, 1986, 1987; Baskin *et al*, 1988). As with HIV exanthem, the virus has not been demonstrated in biopsies obtained from SIV-infected macaques with skin rash (Ringler *et al*, 1986, 1987; Baskin *et al*, 1988). Recently it has been shown that the single or double amino acid substitution (R with Y at position 17 or RQ with YE at positions 17 and 18) in the *nef* gene of the molecular clone SIVmac239 resulted in an exaggerated acute disease syndrome, including earlier onset and more severe maculopapular rash than that observed with wild-type virus (Du *et al*, 1995, 1996; Sasseville *et al*, 1996a).

Here we describe the viral localization and histopathologic and immunophenotypic changes in sequential skin biopsies obtained from six rhesus monkeys infected with these acutely pathogenic clones of SIVmac239. We demonstrate that the onset of rash is earlier than that observed with other strains of SIV and is associated with cellular

infiltrates containing abundant SIV nucleic acid and protein. Perivascular cellular infiltrates within skin biopsies, obtained on the day of rash onset, are composed of equal numbers of CD4+ and CD8+ lymphocytes and abundant αEβ7 positive cells surrounding vessels with upregulated endothelial E-selectin, which is compatible with active recruitment of memory T cells to the skin. Moreover, by examining sequential skin biopsies from the same animal, we show that clearance of virus and resolution of rash is associated with an increase in the percentage of perivascular inflammatory cells expressing CD8, the chemokine receptor CXCR3, and GMP-17, a marker of cytotoxic granules. These results suggest that activated cytotoxic T cells are trafficking to sites of inflammation in the skin and directly or indirectly affect levels of viral replication at these sites.

MATERIALS AND METHODS

Animals and viral infections Eighteen rhesus macaques were infected with either SIVmac239YEnef or SIVmac239/R17Y. Twelve rhesus monkeys were infected with SIVmac239/YEnef (50 ng p27) containing a single NF-κB binding element and two each with SIVmac239/R17Y (300 ng p27) containing zero, one, and two NF-κB elements. The presence of variable numbers of NF-κB elements has no detectable effect on disease course (Du *et al*, 1996). Inoculations were by the intravenous route and two animals from the SIVmac239/YEnef-infected group were killed by an intravenous overdose of sodium pentobarbital at days 3, 7, 14, and 21 post-infection (pi) and three at day 50 pi. Two animals from the SIVmac239/R17Y-infected group were killed by an intravenous overdose of sodium pentobarbital at day 9 and three at day 10. In addition to these time points, one of the SIVmac239/YEnef-infected animals (Mm41–76) and one of the SIVmac239/R17Y-infected animals (Mm204–84) became moribund and were killed at days 12 and 8 pi, respectively. Details of the viral constructs, disease course, pathology, and viral localization in tissue other than skin have been previously published (Du *et al*, 1995, 1996; Sasseville *et al*, 1996a). All animals were negative for antibodies to SIV, Type D retrovirus, and simian T cell leukemia virus type 1 (STLV-1). Animals were housed in accordance with standards of the American Association for Accreditation of Laboratory Animal Care. The investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council.

Virus and virus isolation SIVmac239/YEnef and SIVmac239/R17Y have been described previously (Du *et al*, 1995, 1996). Virus stocks for animal inoculations were produced by transfection of cloned DNA into CEMX174 cells as described in Gibbs *et al* (1994). Peripheral blood was collected prior to inoculation and at days 3, 7, and weekly after inoculation. Infectious virus was isolated from blood by cocultivation of 5×10^6 CEMX174 cells with at least 4×10^6 peripheral blood mononuclear cells as described previously (Desrosiers *et al*, 1989). Culture supernatants were tested biweekly for SIV by p27 capture enzyme-linked immunoassay (Coulter, Miami, FL).

Flow cytometry Two-color flow cytometric analysis of peripheral blood T cell subsets was performed in the SIVmac239/YEnef-infected animals using a whole blood cell lysis technique as described previously (Veazey *et al*, 1997). One milliliter of heparinized blood from each animal was incubated for 45 min at 4°C with monoclonal antibodies against CD4 (OKT4, Ortho Diagnostics Systems, Raritan, NJ) and CD8 (Leu-2a, Becton Dickinson, San Jose, CA) directly conjugated to either fluorescein isothiocyanate, phycoerythrin, or peridinin chlorophyll protein (Becton Dickinson). A gated lymphocyte population was derived from a bivariate histogram display of forward and side scatter and immunofluorescence data for the T cell populations were quantitated using the Cell Quest software package (Becton Dickinson). Absolute CD4 and CD8 cell numbers were calculated by the product of the percentage of CD4 and CD8 cells and the absolute lymphocyte count as determined from a manual count and confirmed by an automated blood analyzer (Serono–Baker 9018, Allentown, PA).

Tissue collection and processing All SIVmac239YEnef-infected animals were examined for the presence of cutaneous rash on days 3, 7, and 14 pi (**Table I**). Animals 41–76 and 247–77 were examined more frequently. All SIVmac239/R17Y-infected animals were examined daily for onset of cutaneous rash. To characterize the progression of cutaneous rash, six animals (one infected with SIVmac239/YEnef and five infected with SIVmac239/R17Y) had sequential skin biopsies taken at the onset of rash and at one or two additional times within the next 24–72 h (**Table II**). For a nonrash control, an additional biopsy was obtained at day 10 pi from the only SIVmac239/R17Y-infected animal that did not develop clinically evident rash (195–84). In addition,

four uninfected animals without evidence of cutaneous disease were biopsied and used as normal skin controls. At least two 8 mm punch biopsies were obtained from each animal while they were anesthetized with Ketamine HCL. Sections were bisected with one-half fixed in 10% neutral buffered formalin for histopathologic examination and the remaining half snap-frozen in OCT Compound (Miles, Elkhart, IN) by immersion in 2-methylbutane cooled in dry ice for immunohistochemistry. For electron microscopy additional biopsies were fixed in 3% gluteraldehyde, postfixed in 1% osmium tetroxide (Stevens Metallurgical, New York, NY), dehydrated, and embedded in eponate 12 resin (Ted Pella, Redding, CA). All sections were stained with uranyl acetate and Reynold's lead citrate and examined on a JEOL 1005 electron microscope.

Localization of SIV-infected cells by *in situ* hybridization and immunohistochemistry *In situ* hybridization was performed with formalin-fixed paraffin-embedded sections by two separate techniques. The first protocol performed by Molecular Histology (Gaithersburg, MD) utilized radiolabeled RNA probes synthesized from five DNA templates covering 90% of the SIV genome subcloned into pGEM4 as previously described (Fox and Cottler-Fox, 1994). The second protocol used a DNA probe labeled with digoxigenin-11-dUTP by random priming (Boehringer Mannheim, Indianapolis, IN) as previously described (Ilyinskii *et al*, 1994; Sasseville *et al*, 1996a). This second technique was combined with immunohistochemistry using the monoclonal antibody HAM-56 (Dako, Carpinteria, CA) for double-labeling SIV-infected macrophages. For these procedures the following controls were included: serial sections of each skin biopsy and known SIV positive and negative tissues were hybridized with sense or anti-sense radiolabeled RNA probes (technique 1), or SIV-specific DNA probes or plasmid pUC19 labeled with digoxigenin-11-dUTP (technique 2).

Sections were examined microscopically and scored semiquantitatively on a scale of 0–4 as follows (**Table II**): the absence of *in situ* positive cells was given a score of 0; 1–5 positive cells per section a score of 1+; 6–15 positive cells per section a score of 2+; 16–30 positive cells per section a score of 3+; and greater than 30 positive cells per section a score of 4+ (**Table II**).

Snap-frozen biopsies were used in immunohistochemical procedures to localize viral antigen as previously described (Horvath *et al*, 1993; Ilyinskii *et al*, 1994). Briefly, tissue sections were fixed in 2% paraformaldehyde immunostained using an avidin-biotin-horseradish peroxidase complex (ABC) technique with diaminobenzidine as the chromogen. The primary antibody used was Senv71.1 (C. Colignon, C. Thiriart, SmithKline Beacham, Belgium), which recognizes SIV gp120.

Immunohistochemistry Formalin-fixed or snap-frozen sections of skin were used to examine the immunophenotype of cellular infiltrates by an ABC immunohistochemical technique as previously described (Horvath *et al*, 1993). T cells and monocyte/macrophages were identified with formalin-fixed tissues using the pan-T polyclonal antibody CD3 (Dako) and the monoclonal antibody HAM-56 (Dako), respectively. Frozen sections were analyzed for T cell subsets using monoclonal antibodies to CD4 (Nu-Th/I, Nicheri Research Institute, Japan) and CD8 (DK 25, Dako). In addition, we examined expression of GMP-17 (TIA-1, Coulter Immunology, Hialeah, FL), which is a marker of cytotoxic granules in cytolytic CD4+ and CD8+ T cells, neutrophils, natural killer cells, and macrophages (Meehan *et al*, 1997).

Expression of select chemokine receptors and adhesion molecules was examined to evaluate the leukocyte recruitment mechanisms responsible for the cellular infiltrates. Chemokine receptor expression was examined in frozen skin sections by an avidin-biotin-alkaline phosphatase complex technique with Fast Red (Biogenex, San Ramon, CA) used as the chromogen as previously described (Rottman *et al*, 1997). Monoclonal antibodies specific for CCR5 and CXCR3 were generated and characterized as previously described (Wu *et al*, 1997; Qin *et al*, 1998).

Relative levels of cellular activation and proliferation were examined by immunostaining for the Ki-67 nuclear proliferation antigen (MIB-1, Immunotech, Westbrook, ME) as previously described (Du *et al*, 1995, 1996; Sasseville *et al*, 1996a). The Ki-67 nuclear antigen is specific for proliferating cells found throughout the cell cycle (G1, S, G2, M phases) and absent in resting (G0) cells (Landberg *et al*, 1990; Barbareschi *et al*, 1994).

Sections immunostained with antibodies directed against CD3, CD4, CD8, HAM-56, GMP-17, and Ki-67 were examined microscopically and scored semiquantitatively on a scale of 0–4 as follows: the absence of immunopositive cells per section was given a score of 0; less than 25% immunopositive cells per section a score of 1+; 25–33% immunopositive cells per section a score of 2+; 33–50% immunopositive cells per section a score of 3+; and greater than 50% immunopositive cells per section a score of 4+ (**Table II**).

Snap-frozen skin sections from all animals were also examined for the adhesion molecules VCAM-1, E-selectin, and αEβ7 (1G11, 1.2B6, and 2G5.1, respectively, Immunotech) by an ABC immunohistochemical technique.

Table I. Onset and duration of SIV exanthem

Virus	Animal #	Days post-infection[a]						
		3	6	7	8	9	10	12
SIVmac239/YEnef	346-80	− |						
	162-78	−						
	400-80	−	−	++ |				
	348-79	−	−	− |				
	41-76	−	−	++++	++++	++++	++++	R |
	39-82	−	−	−	−	−	+	N/E[b]
	191-85	−	−	−	N/E	N/E	N/E	N/E
	189-85	−	−	−	N/E	N/E	N/E	N/E
	457-82	−	−	++++	N/E	N/E	N/E	N/E
	193-85	−	−	−	N/E	N/E	N/E	N/E
	247-79	−	−	++++	++++	++++	++++	N/E
	393-78	−	−	++	N/E	N/E	N/E	N/E
SIVmac239/R17Y	163-91	−			++ |	R	R |	
	205-84	−	++++	++++	++++|			
	168-91	−	−	−	++	++	++ |	
	195-84	−	−	−	−	− |		
	182-91	−	−	−	++++	++++|		
	336-84	−	−	−	++++	R	R |	

[a]A vertical bar represents the day the animal was euthanized and a complete postmortem examination was performed; all animals were clinically examined and rash was subjectively quantified on a scale of 0 to 4 as follows: −, within normal limits; +, minimal; ++, mild; +++, moderate; ++++, severe; R, resolving.
[b]N/E, not examined.

Table II. Immunophenotypic characterization of SIV exanthem

Virus	Animal #		Days post-infection[a]				
			7	8	9	10	12
SIVmac239/YEnef	41-76	Exanthem[b]	++++	+++	++++	++++	R |
		H&E[c]	++++			++++	+++
		SIV RNA[d]	++++			++++	+++
		CD4+/CD8+[e]	++/++				++/++
SIVmac239/R17Y	163-91	Exanthem		++	R	R |	
		H&E		++	++	++++	
		SIV RNA		++	−	−	
		CD4+/CD8+		++/+++	+/++++	++/++++	
	205-84	Exanthem	++++	++++|			
		H&E	++++	++++			
		SIV RNA	+++	++++			
		CD4+/CD8+	+++/+	+++/+			
	168-91	Exanthem		++	++	++ |	
		H&E			+++	+++	
		SIV RNA			+	++++	
		CD4+/CD8+			+++/++	+++/+++	
	195-84	Exanthem	−	−	− |		
		H&E			++		
		SIV RNA			−		
		CD4+/CD8+			+++/++		
	336-84	Exanthem		++++	R	R |	
		H&E		++++	++++	++++	
		SIV RNA		++++	+	+	
		CD4+/CD8+		++/+	++/+	++/++	

[a]A vertical bar represents the day the animal was euthanized and a complete postmortem examination was performed.
[b]All animals were clinically examined and rash was subjectively quantified on a scale of 0 to 4 as follows: −, within normal limits; +, minimal; ++, mild; +++, moderate; ++++, severe; R, resolving.
[c]All animals were examined microscopically and the rash was subjectively quantified on a scale of 0 to 4 as follows: −, within normal limits; +, minimal; ++, mild; +++, moderate; ++++, severe.
[d]All animals were examined microscopically and the rash was subjectively quantified on a scale of 0 to 4 as follows: −, no *in situ* positive cells; +, 1 to 5 positive cells per section; ++, 6 to 15 positive cells per section; +++, 16 to 30 positive cells per section; ++++, greater than 30 positive cells per section.
[e]All animals were examined microscopically and the rash was subjectively quantified on a scale of 0 to 4 as follows: −, no immunopositive cells per section; +, <25% immunopositive cells per section; ++, 25–33% immunopositive cells per section; +++, 33–50% immunopositive cells per section; ++++, >50% immunopositive cells per section.

Although skin specific homing of T lymphocytes occurs through the interaction of E-selectin with cutaneous lymphocyte-associated antigen (CLA) (Picker *et al*, 1991), we did not have an antibody that recognizes this epitope in rhesus monkeys. Thus, as αEβ7 is expressed in 50% of intraepithelial lymphocytes and most αEβ7 positive lymphocytes also express CLA (Spetz *et al*, 1996), we

examined αEβ7 expression in the skin biopsies. Adhesion molecule expression on endothelium and perivascular cells was semiquantitatively assessed by using intensity of immunostaining and distribution of positive cells as criteria as previously described (Silber *et al*, 1994; Sasseville *et al*, 1996c). The numerical values for the distribution of immunoreactive vessels were as follows: 0, none;
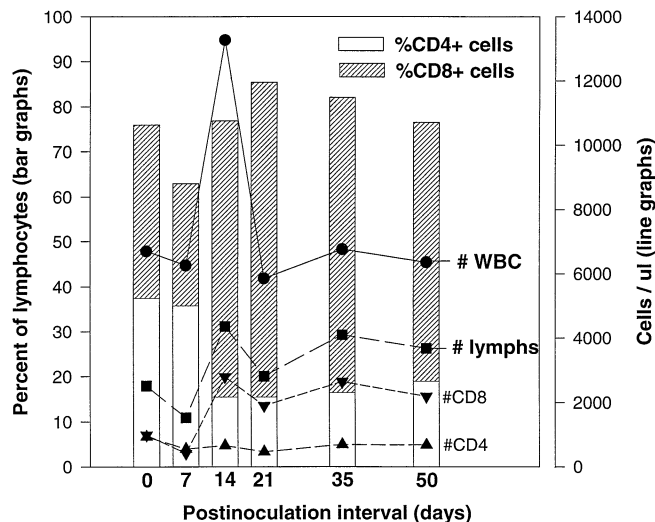
Figure 1. Hematologic values in SIVmac239/YEnef-infected macaques. CD4+ and CD8+ lymphopenia corresponds to peak viral burden in tissues (day 7 pi) followed by CD8+ lymphocytosis on days 14 pi. Data at each time point represent the mean values from all remaining animals (i.e., n = 12 at day 0; n = 2 at day 50). Abbreviations are as follows: #WBC, white blood cell counts; #lymphs, absolute number of lymphocytes; %CD4, CD4+ lymphocytes; % CD8, CD8+ lymphocytes.

1, focal; 2, multifocal; 3, diffuse. The scores for staining intensity were: 0, none; 1, faint; 2, moderate; 3, marked; 4, intense. The product of these two values for distribution and intensity of positive vessels and cellular infiltrates (composite score) had a theoretical range of 0–12.

RESULTS

A mild neutrophilia and CD8 lymphocytosis occur during the first 2 wk of infection   Lymphocyte subset analysis by flow cytometry was limited to the SIVmac239/YEnef-infected animals. These animals had mildly elevated white blood cell counts (13.3×10^3 per μl) peaking at day 14, reflecting a mild transient neutrophilia (8.3×10^3 per μl) (**Fig 1**). In addition, between days 0 and 7 pi mean absolute lymphocyte numbers decreased by 38%, reflecting a decrease in both CD4 and CD8 lymphocytes (**Fig 1**). Between days 7 and 14 pi all remaining animals had a significant rebound in the numbers of CD8 lymphocytes from a mean of 411 per ul (± 265) on day 7 pi to 2789 per μl (± 1312) on day 14, whereas CD4 lymphocytes remained decreased from a mean of 550 (± 170) on day 7 pi to 656 (± 282) on day 14 pi (**Fig 1**). Although absolute CD4 numbers remained relatively unchanged, the percentage of CD4 lymphocytes decreased dramatically due to the marked increase in CD8 lymphocytes (**Fig 1**).

Maculopapular exanthem was observed in most animals by day 8 pi   All animals infected with SIVmac239/YEnef and SIVmac239/R17Y developed an acute disease syndrome characterized by at least one of the following: lethargy, lymphadenopathy, diarrhea, and rash as described previously (Du *et al*, 1995, 1996; Sasseville *et al*, 1996a). The maculopapular rash was observed in five of six animals infected with SIVmac239/R17Y and in six of the 10 animals infected with SIVmac239/YEnef that were still alive after day 7 pi (**Table I**). Severe generalized rash was first observed by day 6 in one animal (Mm205–84) and in three additional animals by day 7 pi. Two other animals at day 7 pi had mild rash limited to the face, neck, and upper torso. By day 8 pi, four additional animals developed either mild localized or severe generalized rash. The last animal to develop rash was 39–82, who developed mild rash limited to the face at day 10 pi. In all cases, the rash had an acute onset and was characterized by small (2–4 mm) to large (1 cm) erythematous macules and papules (**Fig 2**). Two to three days after the onset of rash occasional macules coalesced to form larger variably shaped lesions. Some of the larger macules developed white to yellow crusts within the center of the lesion. Vesicles and ulcers were never observed. Intense erythema lasted for 2–3 d and completely resolved by 4 or 5 d after onset. All of the animals were
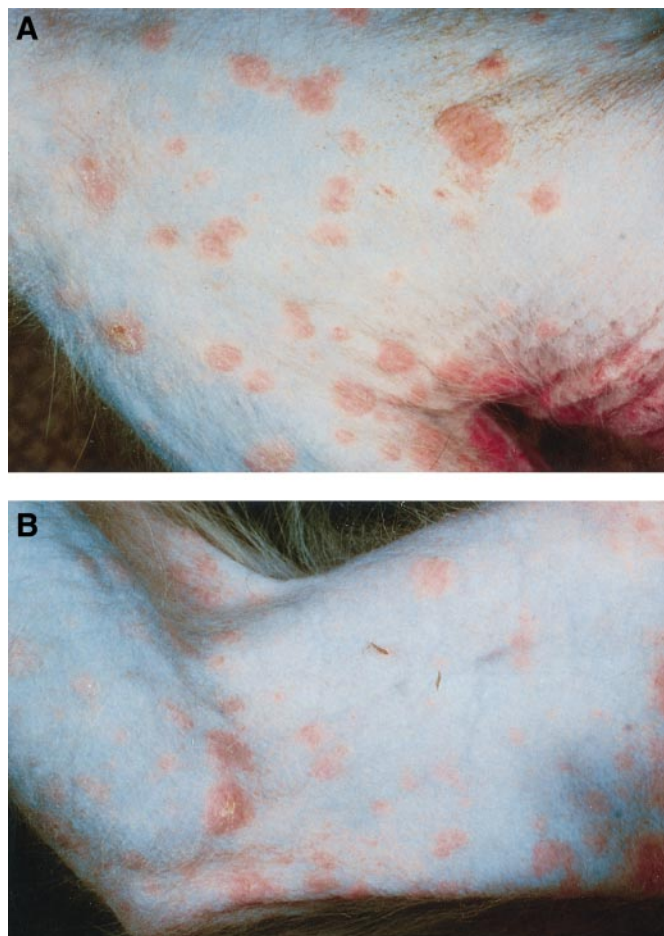

Figure 2. Cutaneous maculopapular exanthem. Ventral axilla and thorax (*A*) and medial aspect of arm (*B*) displaying multifocal erythematous macules and papules at day 10 pi with SIVmac239/YEnef.

viremic with SIV as determined by virus isolation from peripheral blood mononuclear cells.

Histopathology of SIV exanthem resembles HIV exanthem   To examine the development of these lesions, sequential biopsies of skin were obtained (**Table II**). On the day rash was first observed, all biopsies showed similar histopathologic changes characterized by mild to severe focally extensive dermal inflammation, vascular congestion of the superficial vascular plexus, endothelial hypertrophy, and perivascular edema. These infiltrates, which were composed of lymphocytes and macrophages with lesser numbers of neutrophils, were primarily localized to vessels in the superficial dermis. In addition, cellular infiltrates were also occasionally found surrounding hair follicles and multifocally along the base of the overlying epithelium. Perivascular and perifollicular neutrophils were more prominent in biopsies obtained at the onset of rash, but were rare to nonexistent during peak or resolving rash. Generally, the percentage of macrophages increased over time in subsequent biopsies. Where infiltrates came in contact with skin or follicular epithelium, there was mild to moderate acanthosis, spongiosis, exocytosis, dyskeratosis, and parakeratosis; however, neither epithelial vesicles nor ulcers were observed. The inflammatory cell infiltrates and epithelial changes were more intense at the center of the lesion and dissipated toward the tissue margins. Only mild cellular infiltrates were observed in the skin biopsy obtained from the SIV-infected animal without clinically evident rash (195–84, **Table II**), and no significant infiltrates were observed in uninfected control animals.

The development of SIV exanthem is associated with abundant virus positive cells in the dermal perivascular cellular infiltrates   To examine the association of virus with skin rash, we
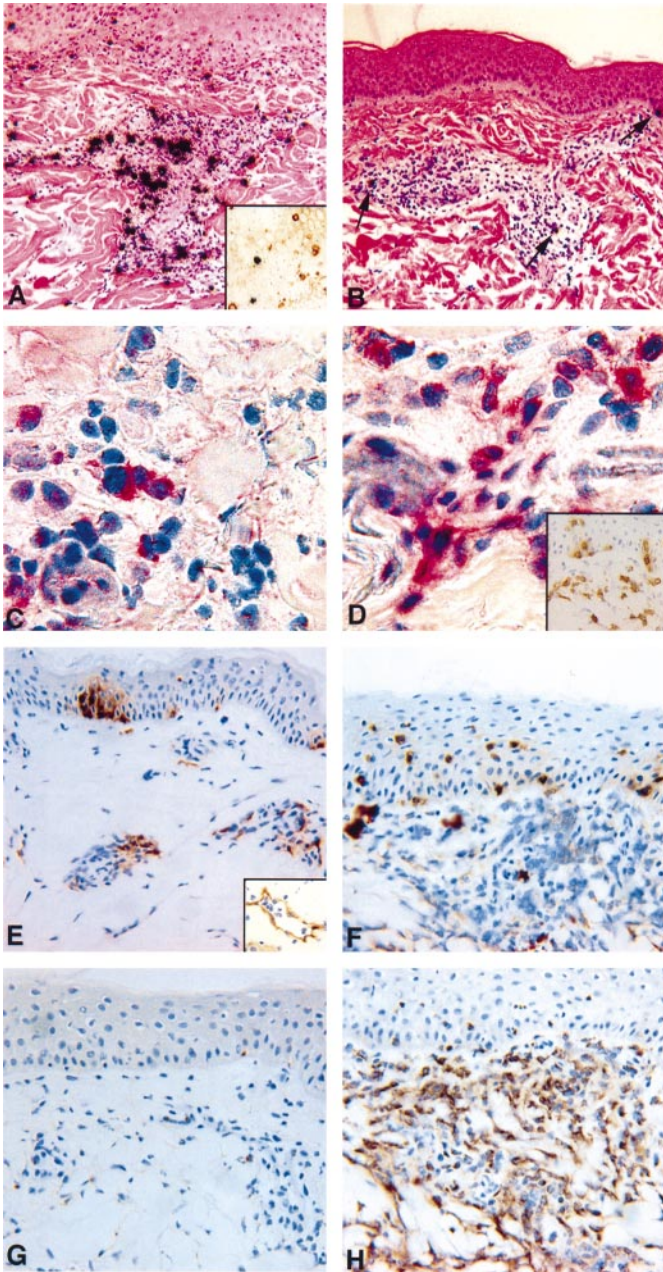
Figure 3. Photomicrographs of SIV exanthem at days 7 (*A, C, E, G*) and 12 (*B, D, F, H*) pi with SIVmac239/YEnef. Abundant SIV positive cells in the perivascular infiltrates from biopsies obtained at rash onset (*A*) *versus* subsequent biopsies (*B, arrows*). Double-labeling for viral mRNA and the macrophage marker HAM-56 revealed that most infected cells (*A, inset,* DAB black) were not colabeled with HAM-56 (*A, inset,* DAB brown). By day 12 pi there was a marked increase in CD8 positive cells (*D, inset*) and in cells expressing the chemokine receptor CXCR3+ (*D*), than that observed at day 7 (*C*). At rash onset, numerous perivascular cells and multifocal aggregates of intraepithelial cells express αEβ7 (*E*). At this time, E-selectin is also upregulated on dermal vessels (*E, inset*). By day 12, most αEβ7 expressing cells are localized to the epithelium (*F*). GMP-17 is absent in the αEβ7 cells trafficking to the skin at rash onset (*G*), but is expressed on most of these perivascular cells by day 12. (*A, B*) *In situ* hybridization with hematoxylin and eosin counterstain; (*C, D*) ABC technique with Fast Red chromogen and hematoxylin counterstain; (*D inset*) *in situ* hybridization with DAB black chromogen and ABC immunohistochemical technique with DAB brown chromogen and hematoxylin counterstain; (*E, E inset, F, G, H*) ABC technique with DAB brown chromogen and hematoxylin counterstain.

performed *in situ* hybridization and immunohistochemistry for viral nucleic acid and antigen in six of the SIV-infected animals. Five of these animals had rash evident grossly and one (195–84) never developed rash.

In SIV-infected animals with cutaneous rash, abundant *in situ* positive cells were first observed in the initial biopsies obtained at either day 7 or 8 pi (**Table II**). Most of the positive cells were found in the perivascular infiltrates of the superficial dermis (**Fig 3***A*).Occasional SIV-RNA positive cells were observed within the epidermis overlying areas of dermal inflammation (**Fig 3***A*). Double-labeling for viral mRNA and the macrophage marker HAM-56 revealed that most SIV positive cells did not colabel with the macrophage marker, suggesting that most infected cells were lymphocytes (**Fig 3***A, inset*). Immunohistochemical expression of gp120 in adjacent skin sections correlated with viral mRNA expression, but generally fewer positive cells were detected by immunohistochemistry than by *in situ* hybridization (data not shown). Virus was not detected in any of the biopsies by electron microscopy. Despite mild cellular infiltrates in the dermis of the animal without clinical rash (195–84), virus was not detected by *in situ* hybridization, immunohistochemistry, or electron microscopy. In four animals, a second biopsy was obtained within 24 h of collecting the initial biopsy. In two of these animals (163–91, 336–84), viral burden in the skin, as assessed by semiquantitative microscopic examination, was substantially decreased or absent. Two of these four animals (168–91, 205–84) had increased viral burden within 24 h of the initial biopsy. As both of these animals were killed within 48 h of the onset of rash, we were unable to assess the progression of rash in these animals.

Cellular infiltrates consist primarily of cytotoxic T cells   To immunophenotype the cellular infiltrates, all biopsies were immunostained with antibodies directed against the T lymphocyte markers CD3, CD4, and CD8, and the macrophage marker HAM-56. In addition, we examined the expression of GMP-17, which is a marker of cytotoxic granules, and Ki-67, a nuclear proliferation antigen. In animals with rash, the percentage of CD3+ lymphocytes in perivascular and perifollicular infiltrates varied between 25% and > 50% (data not shown). No significant differences in the percentages were observed among animals or among biopsies obtained at various times after the onset of rash. Intraepithelial CD3+ cells were most abundant (5–10 per hpf) above dermal infiltrates. In most skin biopsies examined, the percentage of HAM-56 positive cells in these lesions increased from less than 25% on initial biopsy to ≈50% in subsequent samples (data not shown). Unlike CD3+ lymphocytes that were restricted to the epithelium overlying dermal infiltrates, numerous intraepithelial HAM56+ cells were observed throughout the epidermis.

Depending on the biopsy examined, the relative proportions of mononuclear cells immunoreactive with antibodies against CD4 and CD8 varied considerably among animals (**Table II**). Among biopsies from different animals, the range of CD4+ and CD8+ cell counts was 25% to 50% and < 25% to > 50%, respectively; however, when sequential biopsies from the same animal were compared, there was no significant difference in CD4+ cell counts, but in most animals a marked increase in CD8+ lymphocytes was observed between the initial and subsequent biopsies (**Table II, Fig 3***D, inset*). GMP-17 expression was absent in normal skin and in biopsies obtained at the onset of rash (**Fig 3***G*); however, GMP-17 immunopositive cells comprised > 50% of the perivascular cells in subsequent biopsies, suggesting an influx of cytotoxic cells during the resolution of rash (**Fig 3***H*).

Cellular infiltrates in SIV exanthem are recruited and proliferate *in vivo*   CXCR3 is expressed predominantly on activated CD8+ lymphocytes (Qin *et al*, 1998). Thus, as with the percentage increase in CD8+ lymphocytes, expression of the chemokine receptor CXCR3 increased between the initial and subsequent biopsies obtained from animals with rash (**Fig 3***C, D*). CXCR3 expression was not observed in control skin from uninfected animals. In these same biopsies, CCR5 expression was generally observed in < 25% of the perivascular inflammatory cells, but there was no significant difference between the initial and subsequent biopsies (data not shown). CCR5 expression

was elevated over the minimal expression observed in control skin from uninfected animals.

E-selectin expression was moderate to markedly elevated on endothelium in most dermal and subcutaneous vessels in all biopsies examined on the day of rash onset (**Fig 3E**, *inset*), but decreased in most animals thereafter (data not shown). No E-selectin was observed in normal skin. We did not have an antibody that recognizes CLA, the ligand for E-selectin in the skin, but the integrin αEβ7 is coexpressed with CLA in intraepithelial lymphocytes (Spetz *et al*, 1996). In normal skin the expression αEβ7 was restricted to rare intraepithelial lymphocytes, but in biopsies taken at the onset of rash, αEβ7 expression was observed in ≈50% of the perivascular cells and in many of the intraepithelial lymphocytes (**Fig 3E**). In subsequent biopsies αEβ7 expression was localized to intraepithelial lymphocytes, but was decreased in perivascular inflammatory cells (**Fig 3F**). Thus, αEβ7 expression on perivascular inflammatory cells coincided with peak upregulation of E-selectin on dermal vessels (rash onset), and decreased thereafter.

In contrast to the diffuse E-selectin expression, endothelial VCAM-1 expression was localized to focal vessels within the superficial dermis. In addition to endothelium, moderate to marked VCAM-1 expression was observed on cells morphologically compatible with dendritic cells and perivascular macrophages within the dermis in all samples (data not shown).

Ki-67 immunoreactivity in unaffected skin adjacent to dermal lesions was localized to isolated epithelial cells in the stratum basale and external root sheath of hair follicles with few or no positive cells in the dermis. In areas with dermal inflammation, the overlying acanthotic epithelium had intense and diffuse expression on all cells within the stratum basale and in the external root sheath of hair follicles and the germinative zones of sebaceous glands (data not shown). Twenty-five to 50% of the perivascular infiltrates were immunopositive for Ki-67, suggesting *in situ* proliferation. There was no correlation between percentage of cells immunopositive for Ki-67, time pi, intensity of inflammation, or presence of viral RNA or protein.

DISCUSSION

In this study, we utilized the SIV-infected macaque model of AIDS to investigate the pathogenesis of the exanthem associated with acute HIV-1 infection. The rash observed in SIVmac239/YEnef and SIVmac239/R17Y-infected animals consisted of mild-to-moderate vascular congestion in the superficial vascular plexus, endothelial hypertrophy, perivascular edema, and lymphocytic perivascular cuffs similar to that described in detail previously for HIV and SIV exanthem (Ringler *et al*, 1986, 1987; McMillan *et al*, 1989; Balslev *et al*, 1990; Hulsebosch *et al*, 1990; Clark *et al*, 1991; Lapins *et al*, 1996). Sequential skin biopsies were obtained from rhesus monkeys with rash resulting from infection with SIVmac239/YEnef and SIVmac239/R17Y. In biopsies obtained at the onset of rash, we observed elevated expression of E-selectin, abundant SIV nucleic acid and protein, and αEβ7 positive perivascular lymphocytes compatible with increased recruitment of activated T cells to the skin very early after infection with SIV. Moreover, we demonstrated that clearance of virus from the skin and resolution of rash were associated with an increase in inflammatory cells expressing CD8, cytotoxic granules (GMP-17), and the chemokine receptor CXCR3.

Although viremia is associated with the onset of HIV exanthem (Clark *et al*, 1991; Daar *et al*, 1991), the direct role of dermal invasion by HIV in the pathogenesis remains speculative (McMillan *et al*, 1989; Duvic, 1995; Lapins *et al*, 1996). In fact, there is only a single report in the literature demonstrating HIV p24 antigen in perivascular inflammatory cells from a patient with rash arising 10 d after HIV-1 exposure (McMillan *et al*, 1989). Likewise, studies in macaques infected with other isolates of SIV failed to detect virus within the epithelium or in the perivascular inflammatory cells (Ringler *et al*, 1986, 1987; Baskin *et al*, 1988). In contrast, we demonstrated abundant SIV in the dermal infiltrates, which supports the role of virus-infected cells in the development of cutaneous rash. Moreover, resolution of the rash is associated with decreased cutaneous viral burden, despite abundant viral load in these same animals in the gastrointestinal tract and

lymphoid tissues (Du *et al*, 1995, 1996; Sasseville *et al*, 1996a). Thus, the presence of virus in the skin most likely precipitates clinically evident cutaneous exanthem in SIV-infected macaques.

We hypothesize that there is increased recruitment of activated lymphocytes to the skin and localized lymphocyte proliferation (Ki-67+) serving as a source for viral replication during acute stages of SIV infection. Early in HIV and SIV infection, virus localizes in lymphoid tissues, which have an abundant source of activated target cells (Biberfeld *et al*, 1986; Chakrabarti *et al*, 1994; Lackner *et al*, 1994). Similar to lymph node and gastrointestinal lymphoid tissues, the skin contains antigen presenting cells (dendritic cells), cytokine producing cells (keratinocytes), and skin tropic T cells of the memory phenotype supporting the concept of skin-associated lymphoid tissue (Streilein, 1990; Memar *et al*, 1995). Moreover, the skin contains abundant microvessels within the superficial dermis, which have been shown to express adhesion molecules and chemokines imperative for directed cell trafficking (Walsh *et al*, 1990; Picker *et al*, 1991; Silber *et al*, 1994; Rand *et al*, 1996). In this regard, E-selectin has been shown to be an important adhesion molecule in the homing of CLA positive memory T cells to sites of cutaneous inflammation (Picker *et al*, 1991). Thus, similar to the mucosal addressin MAdCAM and the peripheral lymph node vascular addressin PNAd (Streeter *et al*, 1988), E-selectin serves as a skin vascular addressin (Picker *et al*, 1991). In agreement with the findings of Silber *et al* (1994), demonstrating E-selectin expression in two different cutaneous inflammatory models in rhesus monkeys, we also observed prominent E-selectin expression on dermal vasculature in all of the skin biopsies taken from animals at onset of rash. We were unable to confirm the presence of CLA on the perivascular T cell infiltrates in these tissues as the murine antibodies directed against the human CLA antigen did not recognize the epitope in rhesus monkeys, but these cells did express αEβ7 that is coexpressed with CLA on intraepithelial lymphocytes (Spetz *et al*, 1996). Thus, the presence of αEβ7 positive cells associated with peak endothelial E-selectin in biopsies obtained at the onset of rash suggest that, as in lymph node and gastrointestinal lymphoid tissues, there is increased recruitment of activated lymphocytes to the skin in response to antigenic stimuli during acute SIV infection.

Whether or not there is selective recruitment of viral infected lymphocytes or infection of CD4+ cells once they arrive at the sites of inflammation cannot be determined from this study; however, the multifocal distribution of isolated virus positive cells in the perivascular infiltrates suggests the former. Previous observations by us have suggested that virus positive cells readily traffic to sites of preexisting inflammation early after SIV infection (Sasseville *et al*, 1996b), but do not during late-stage disease (Horvath *et al*, 1993; Sasseville *et al*, 1995). In addition to increased recruitment of lymphocytes to the skin, the presence of abundant Ki-67 immunopositivity suggests lymphocyte proliferation at these sites. The finding of increased endothelial adhesion molecules and Ki-67 supports our previous hypothesis that SIV mac239YEnef-induced lesions are the result of increased recruitment and also proliferation of leukocytes providing a continuous source of activated cells available for viral replication (Sasseville *et al*, 1996a).

In addition to elevated adhesion molecule expression on microvascular endothelium, leukocytes are also activated and migrate in response to chemotactic gradients elicited from inflammatory sites (Springer, 1994). The chemotactic stimuli responsible for lymphocyte recruitment to the skin in HIV/SIV exanthem are unknown, but select chemokines have been shown to be elevated in tissues with HIV and SIV-induced inflammation (Sasseville *et al*, 1996c; Schmidtmayerova *et al*, 1996; Tedla *et al*, 1996; Zou *et al*, 1997). The CXC chemokines interferon-inducible protein-10 (IP-10) and Mig are potent chemoattractants of activated T cells via the chemokine receptor CXCR3 (Loetscher *et al*, 1996), and IP-10 has been shown to be expressed on interferon-γ-induced microvascular endothelial cells (Goebeler *et al*, 1997). Thus, we examined these infiltrates for immunohistochemical expression of CXCR3. Between the time of initial biopsy taken at onset of rash and subsequent samples, we found that there was a marked increase in the percentage of CXCR3 positive cells. Although double labeling for CD8+ and CXCR3+ in skin biopsies was not done, we have demonstrated that CXCR3 is coexpressed on CD8+ T cells and

absolute numbers of CD8+ lymphocytes and CXCR3+ lymphocytes rise in parallel in peripheral blood and lymph nodes in rhesus monkeys infected with the pathogenic molecular clones SIVmac239 and SIVmac239/316 (data not shown). Moreover, we observed an association between increased perivascular cells expressing GMP-17 and resolution of rash. It is also known that a rapid cytotoxic T lymphocyte response occurs within days of SIV and HIV-1 infection and is associated with a decrease in viral loads (Reimann *et al*, 1991 , 1994; Jassoy *et al*, 1992; Yasutomi *et al*, 1993; Joag *et al*, 1994; Safrit *et al*, 1994; Gallimore *et al*, 1995). In this study, SIVmac239YEnef-infected animals had a 2-fold rebound in the absolute number and percentage of CD8+ peripheral blood lymphocytes between days 7 and 14 pi. Interestingly, this is the time in which increased CD8+ and CXCR3+ lymphocytes were observed in the skin infiltrates. This early peak in CD8+ lymphocytes is unique in that peak lymphocytosis is generally observed around day 21 pi with other pathogenic isolates of SIV (Reimann *et al*, 1994). These results suggest that activated CXCR3+ T cells readily traffic to the skin and may directly or indirectly affect levels of viral replication at these sites.

In addition to their roles in chemoattraction, certain chemokine receptors have recently been shown to serve as coreceptors along with CD4 for infection of target cells by both HIV and SIV (Alkhatib *et al*, 1997; Chen *et al*, 1997; Deng *et al*, 1997; Edinger *et al*, 1997; Marcon *et al*, 1997). Because one of these chemokine receptors termed CCR5 is utilized by macrophage-tropic (M-tropic) HIV-1 strains (Alkhatib *et al*, 1996; Choe *et al*, 1996; Deng *et al*, 1996; Dragic *et al*, 1996), as well as both M-tropic and T cell tropic strains of SIV (Chen *et al*, 1997; Edinger *et al*, 1997; Marcon *et al*, 1997), we examined its expression in the skin. CCR5 expression was elevated over that observed in normal skin from uninfected animals, but never reached the level of expression of CXCR3. As it has been shown that CCR5 is expressed by macrophages and the effector/memory subset of T cells (Wu *et al*, 1997), it is possible that enhanced recirculation of previously activated lymphocytes from the peripheral blood to the skin may occur early in SIV infection. This pool of activated CD4+ cells expressing CCR5 may serve as a source for early rounds of viral replication. In addition to CCR5, it has been shown that M-tropic HIV-1, HIV-2, and SIV also use two newly described G-protein-coupled receptors, termed Bonzo (STRL33) and BOB (Alkhatib *et al*, 1997; Deng *et al*, 1997). The expression of these two novel receptors has not been examined in the skin.

By using the molecular clones of SIVmac239/YEnef and SIVmac239/R17Y that cause an exaggerated acute disease syndrome in rhesus monkeys, we were able to unravel some of the mysteries concerning the pathogenesis of HIV exanthem. The increased expression of E-selectin, a putative skin-associated lymphoid tissue addressin, and the abundance of αEβ7 perivascular lymphocytes, suggest that there is increased recruitment of activated T cells to the skin very early after infection. The presence of activated CD4+/CCR5+ lymphocytes along with increased local lymphoid proliferation (Ki-67 positive), may serve as the nidus for viral invasion of the skin similar to that observed in lymph nodes and gut associated lymphoid tissue. In biopsies obtained during resolution of rash, the increase in CD8+, CXCR3+, and GMP-17+ cells trafficking to the skin is likely to be responsible for the decreased viral burden in the skin.

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