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In situ hybridization and immunolabelling study of the early replication of simian immunodeficiency virus (SIVmacJ5) *in vivo*

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The distribution of virus-infected cells in cynomolgus macaques was determined at 4, 7, 14 and 28 days following intravenous challenge with 1000 $TCID_{50}$ of the wild-type simian immunodeficiency virus SIVmacJ5 (stock J5C). At each time-point, pairs of macaques were killed humanely and the presence of SIV was determined and quantified in blood, spleen, peripheral and mesenteric lymph nodes, thymus, lung and ileum by virus co-cultivation with C8166 cells, by quantitative DNA PCR or by *in situ* hybridization (ISH). At day 4 post-infection (p.i.), detection of the virus was sporadic. By day 7 p.i., however, significant SIV loads were detected in the blood and lymphoid tissues by DNA PCR and virus co-cultivation. Large numbers of cells expressing SIV RNA were detected in mesenteric lymph nodes by ISH and significantly fewer ($P < 0{\cdot}05$) in the spleen. Significant numbers of ISH-positive cells were also observed in sections of ileum. By day 14 p.i., the distribution of SIV was more even in all lymphoid tissues analysed. By day 28, most of the tissues were negative by ISH, but all remained positive by virus isolation and DNA PCR. Immunolabelling of sections of mesenteric lymph node with monoclonal antibodies specific for SIV envelope and Nef largely confirmed the observations from ISH. These results indicate that, even following intravenous challenge, a major site of the initial replication of SIV is gut-associated lymphoid tissue. Vaccines that induce protection at this site may therefore be superior, even against parenteral challenge.

Introduction

The initial interactions upon infection between human immunodeficiency virus (HIV) and its host are critical in determining the clinical outcome of infection. The application of highly sensitive assays to quantify virus loads in plasma has demonstrated that the kinetics of the primary viraemia and the establishment of virus set-point are important determinants of disease progression (Cao *et al.*, 1995; Mellors *et al.*, 1996). However, it is believed that the viruses present in the blood are derived from productively infected cells located in lymphoid organs, rather than from PBMC (Simmonds *et al.*, 1991). Thus, characterization of the early pathogenesis of HIV infection in lymphoid tissues should provide insights into the interplay between the virus and the host and identify factors that may be crucial in determining the clinical prognosis of infection. The difficulties of obtaining relevant clinical material from these early stages in HIV infection (Schacker *et al.*, 2000) means that we must turn to animal models to investigate the early pathology of lentivirus infection. The experimental infection of macaques with simian immunodeficiency virus (SIV) is a valuable model of HIV infection and AIDS in man (Stott & Almond, 1995; Almond & Heeney, 1998). We have used the experimental infection of cynomolgus macaques with wild-type virus, SIVmac32H clone J5 (SIVmacJ5; Rud *et al.*, 1994), as a model for studying the distribution of virus in lymphoid tissues during the first 4 weeks following intravenous challenge. Virus co-culture, DNA PCR, *in situ* hybridization (ISH) and immunohistochemistry were used to detect SIV in each tissue compartment. These studies revealed that gut-associated

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lymphoid tissue is the predominant initial site of virus replication, and the virus is later disseminated to other lymphoid tissues. It is important that these observations are considered in the development of novel immunization vectors and protocols designed to improve vaccine efficacy.

Methods

■ **Animals.** The study group consisted of ten juvenile cynomolgus macaques (*Macaca fascicularis*), which were purpose bred in Europe. The animals were housed and maintained according to Home Office (UK) guidelines for the care and maintenance of primates. Animals were sedated with ketamine hydrochloride before inoculation of virus or venepuncture and killed humanely by an overdose of anaesthetic.

■ **Virus.** The challenge virus was derived from the molecular clone SIVmac32H (J5) (Rud *et al.*, 1994). The virus was derived from the 3/92 stock J5M (Cranage *et al.*, 1997) by passaging on lymphocytes from a single uninfected cynomolgus macaque. The virus stock referred to as J5C was titrated *in vitro* on C8166 cells and had an end-point titre of 10^3 $TCID_{50}$/ml. Based on the titration, a dose of 10^3 $TCID_{50}$ was used to challenge all macaques in this study.

■ **Plan of study.** Two experiments were performed in this study. In the first, six macaques, N17–N22, were challenged by intravenous inoculation of 1000 $TCID_{50}$ of the J5C virus stock. Pairs of macaques were killed humanely at 7 (N17, N18), 14 (N19, N20) and 28 (N21, N22) days after challenge and the presence of virus was determined in blood, inguinal, axillary and superior mesenteric lymph nodes, spleen, thymus and lungs. In the second experiment, four macaques, P213–P216, were challenged with 1000 $TCID_{50}$ of virus and pairs of macaques were sacrificed at 4 (P213, P214) and 7 (P215, P216) days after challenge; the presence of virus was determined in the ileum in addition to the lymphoid tissues studied previously.

■ **Detection of virus by co-culture and DNA PCR.** Re-isolation of virus from PBMC or lymphocytes recovered from disrupted lymphoid tissues was achieved by co-cultivation with C8166 cells. Cultures were maintained for 28 days and virus was detected by the appearance of syncytia and confirmed with an SIV p27 antigen-capture assay (Rose *et al.*, 1995). Virus loads were determined by co-cultivating dilutions of PBMC or lymphocytes containing between 5×10^6 and 10^2 cells with indicator cells as described above.

SIV DNA was detected in blood or tissue by specific amplification of a region within the *gag* gene using PCR and nested primers, as described previously (Rose *et al.*, 1995). Quantification of SIV DNA load was determined by an end-point dilution as described previously (Slade *et al.*, 1995) except that specific amplification of a 147 bp portion of the SIV *gag* gene was achieved by a single round of PCR with primers SG131N (5′ TTGGATTAGCAGAAAGCCTG 3′) and SG277C (5′ TCTCTTCTG-CGTGAATGCAC 3′) with Amplitaq Gold (PE Applied Biosystems) and thermocycling conditions of 94 °C for 10 min followed by 40 cycles of 94 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s and finished with 72 °C for 10 min. Following agarose gel electrophoresis and transfer to nylon membrane (Hybond-N, Amersham) by Southern blotting, specific PCR products were identified by hybridization with the ^{32}P-end-labelled oligonucleotide SG176N (5′ AATACTTTCGGTCTTAGCTC 3′) as described previously (Rose *et al.*, 1995).

■ **Serological assays.** Anti-SIV envelope antibodies in plasma were detected by ELISA, performed with recombinant SIV gp140 (Repligen, ADP625.1) as antigen, as described previously (Kent *et al.*, 1994). Neutralizing activity in serum was determined as described previously (Kent *et al.*, 1994).

■ **Preparation of digoxigenin-labelled DNA probes for ISH.** Single-stranded DNA probes labelled with digoxigenin were prepared by using asymmetric nested PCR as described by An *et al.* (1992). Briefly, three pairs of oligonucleotide primers were used to amplify by PCR regions of the SIV *gag* (SG1411N, 5′ GAAACTATGCCAAAAACA-AGT 3′; SG2154C, 5′ TAATCTAGCCTTCTGTCCTGG 3′), *env* (SE7054N, 5′ GCACAGGCTTGGAACAAG 3′; SE7695C, 5′ AGTTC-CAGTATACCTGGGATG 3′) and *nef* (SN9500N, 5′ AGACATGTAC-TTAGAAAAGGA 3′; SN9866, 5′ TCAGCGAGTTTCCTTCTTGT 3′) genes. Following purification of PCR products using glassmilk (Geneclean II, Bio101) and quantification, approximately 60 ng product from the first-round amplification was transferred to a second round of 40 cycles of amplification containing a single oligonucleotide primer and a dNTP mix containing digoxigenin-labelled dUTP (Boehringer Mannheim).

Two probes were prepared from each product of the first round of amplification. Probes that were identical to sequences within SIV transcripts were produced by using primers SG1829N (5′ CAG GAG ATG GAT CCA ACT 3′), SE7386N (5′ GAG ACA CAG ACT TCT ACT TGG 3′) and SN9641N (5′ TGC AGC CAG CTC AAA CTT C 3′). Probes that were complementary to SIV transcripts were obtained by using primers SG1617C (5′ ATA GGG GAG GCA GCC TTC TGA CAG 3′), SE7305C (5′ CAA AGC ATA ACC TGG AGG TGC 3′) and SN9763C (5′ GGG TAT CTA ACA TAT GCC TC 3′). Incorporation of digoxigenin–dUTP into each final product was assessed by end-point dilution and materials supplied in the DIG nucleic acid detection kit (Boehringer Mannheim) following the manufacturer's instructions.

■ ***In situ* hybridization (ISH).** Tissue samples were immersed in 10% (v/v) formal saline for 24–96 h at 4 °C before processing. Sections (4 μm) of paraffin wax-embedded tissues were mounted onto glass slides coated with 3-aminopropyl triethoxysilane (APES, Sigma). Prior to hybridization, sections were dewaxed, rehydrated and permeabilized with proteinase K (Boehringer Mannheim) for 30 min at 37 °C before refixing and dehydration in ethanol.

Hybridization of digoxigenin-labelled probes was performed with the Omnislide system (Hybaid). Labelled probes were diluted up to 1:40 and a cocktail of all three probes that were normal to or three probes that were complementary to SIV transcripts was mixed in hybridization buffer [2 × SET (50 mM Tris–HCl, pH 7·4, 300 mM NaCl, 2 mM EDTA) containing 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0·05% (w/v) SDS, 0·05% (w/v) PVP and 500 μg/ml boiled, sonicated salmon sperm DNA] and allowed to hybridize by incubation overnight at 37 °C. Following hybridization, slides were washed in hybridization buffer containing 0·1% (v/v) Triton X-100 at 42 °C for 3 min.

The location of digoxigenin-labelled probes was visualized using an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Boehringer Mannheim) and NBT/BCIP solution (Gibco-BRL) as chromogenic substrate following conditions recommended by the manufacturers. Sections were counterstained with neutral red and mounted in Glycergel (Vector Laboratories).

In order to evaluate the comparative sensitivity of this method with that using ^{35}S-labelled RNA probes, we sent sections consecutive to those analysed in this study to T. Reinhart (formerly of Glaxo Wellcome, Stevenage, UK, now of the University of Pittsburgh, USA). Using methods described previously (Reinhart *et al.*, 1997), the frequency and distribution of hybridization-positive cells were indistinguishable when determined by the two methods (T. A. Reinhart, personal communication).

■ **Analysis of ISH.** For each section, the area of lymphoid tissue present was calculated by outlining the relevant region from low-power micrographs mounted on a digitizing tablet (Kontron Digiplan). ISH-positive cells were enumerated independently by two people. The

statistical difference of the density of hybridizing cells (number of positive cells/mm^2) was assessed by the non-parametric Mann–Whitney U-test.

■ **Immunolabelling of SIV Gag, Nef and envelope proteins in histological sections.** Sections (4 μm) from paraffin wax-embedded tissue were mounted onto 3-APES-coated slides, dewaxed, dehydrated and incubated in ethanol containing 0·5% (v/v) hydrogen peroxide (BDH) at room temperature for 20 min to block endogenous peroxidase activity. The slides were immersed in 10 mM citrate buffer (pH 6·0) and 'cooked' at high power in a microwave oven on two occasions for 5 min. Once the sections had cooled to room temperature, they were rinsed in TBS (20 mM Tris–HCl, pH 7·5, 225 mM NaCl) and immersed in TBS containing 5% (w/v) non-fat milk powder for 60 min at room temperature. Monoclonal antibodies (MAbs) of the IgG1 isotype to SIV Gag p17/p27 (KK59/KK64; Kent *et al.*, 1991), envelope (KK13; Kent *et al.*, 1991) and Nef (KK75; Arnold *et al.*, 1999) were respectively diluted 1:50, 1:2 and 1:50 in TBS containing 1% (w/v) BSA, added to the section and incubated overnight at 4 °C. After extensive washing in TBS, bound antibodies were visualized by using a biotinylated universal horse anti-mouse/rabbit antibody (Vector Laboratories) and an avidin/biotinylated horseradish peroxidase complex (ABC, Vector Laboratories) using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as chromogenic substrate. Sections were counterstained with haematoxylin (R. A. Lamb) and mounted in DPX (R. A. Lamb).

Results

Outcome of virus challenge

Following intravenous inoculation with SIVmacJ5, all macaques became infected, as determined by virus isolation and/or DNA PCR (see Table 1). The two macaques killed at 4 days post-infection (p.i.) differed in virus recovery. Macaque P213 was SIV-positive in spleen and peripheral lymph nodes by both co-cultivation and DNA PCR. In addition, samples of PBMC were positive by co-culture only and mesenteric lymph node by PCR only. In contrast, macaque P214 was SIV-negative by DNA PCR and only virus co-culture-positive in blood and spleen by the co-culture of 5×10^6 lymphocytes with indicator cells. By days 7, 14 and 28 p.i., SIV was recovered from all tissues analysed by both DNA PCR and virus co-culture, with the exception of the co-culture of spleen cells from macaque N17. Where virus load was quantified by both co-culture and DNA PCR in a single macaque, there was no clear correlation between the results of these two assays. At each time-point studied, there was considerable variation between individual animals but, overall, a picture of extensive virus replication between day 7 and 28 emerged. The highest virus loads were detected by DNA PCR from the spleens of macaques N19 and N20 at day 14 p.i. More limited studies by DNA PCR to detect SIV in the thymus and lungs of macaques also showed a similar picture, detecting a consistent peak at day 14 p.i. All macaques seroconverted to recombinant SIV gp140 by day 14 p.i. (range of end-point ELISA titres, 2·1–3·3 $\log_{10}$), but no neutralizing activity (titre $< 1/10$) was detected in serum at this time. By 4 weeks p.i., N21 and N22 had end-point ELISA titres of $> 4{\cdot}0$ $\log_{10}$ and also had significant neutralizing antibody ($\log_{10}$ titres: N21 = 2·8; N22 = 2·1).

Table 1. Recovery of virus by co-cultivation and DNA PCR from blood and tissues of SIVmacJ5-infected macaques

Columns headed VI give the virus load, presented as the number of SIV-positive cells per 10^6 cells. Where VI is not quantified: −, virus load < 1 per 5×10^6 lymphocytes; +, virus load > 1 per 5×10^6 lymphocytes. Columns headed PCR give the DNA load, presented as the number of copies of template per 10^6 genome equivalents. PLN, Peripheral lymph node; MLN, mesenteric lymph node; ND, assay not performed.

Macaque	Time (days p.i.)	Blood		Spleen		PLN		MLN		Thymus		Lung		Ileum	
		VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR
P213	4	3	< 30	3	3	3	1000	< 0·2	220	ND	ND	ND	< 32	ND	< 300
P214	4	< 3(+)*	< 1800	< 3(+)*	< 60	< 0·2	< 600	ND	< 62	ND	ND	ND	< 84	ND	< 600
P215	7	100	880	320	130	100	80	ND	230	ND	ND	ND	30	ND	19
P216	7	320	1390	1000	1400	320	15	3200	690	ND	ND	ND	60	ND	8900
N17	7	+	660	−	2500	ND	720	+	5600	ND	2	ND	250	ND	ND
N18	7	+	4200	+	630	ND	320	+	2900	ND	2	ND	60	ND	ND
N19	14	100	2200	320	55000	+	2000	+	8900	ND	2000	ND	1100	ND	ND
N20	14	3200	4700	320	18500	+	3700	+	1850	ND	6000	ND	1000	ND	ND
N21	28	320	1250	320	1700	+	3000	+	3300	ND	150	ND	50	ND	ND
N22	28	320	90	32	450	+	3300	+	440	ND	670	ND	10	ND	ND

* Outcome of virus co-culture of 5×10^6 lymphocytes with C8166 cells.

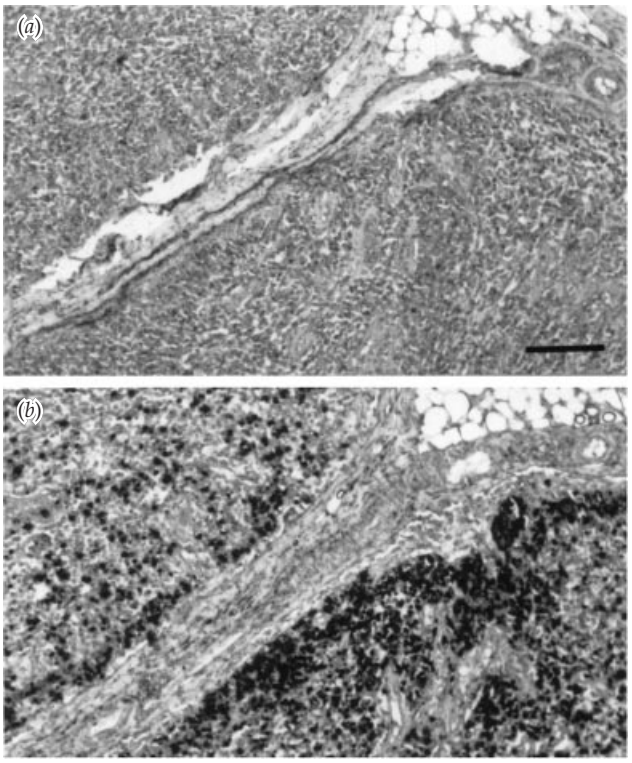

Fig. 1. Comparison of the binding of normal (*a*) and complementary (*b*) single-stranded digoxigenin-labelled DNA probes to consecutive sections of a formalin-fixed, paraffin wax-embedded peripheral lymph node removed from a macaque 73 days after challenge with SIV, when there were symptoms of incipient AIDS. The micrographs are at the same magnification. Bar, 100 μm.

Histopathological studies

Histopathological examination of the lymphoid tissues collected post mortem revealed mild follicular hyperplasia. This was most marked in mesenteric lymph nodes at 7, 14 and 28 days p.i., but it was not possible to associate the changes directly with infection by SIV.

ISH

A non-radioactive ISH technique was established to determine the relative position of SIV-infected cells within lymphoid tissues. Prior to the analyses of tissues from macaques in the study, the relative ability of the protocol to detect viral transcripts and virus RNA from double-stranded SIV DNA was assessed. Digoxigenin-labelled normal and complementary strand probes were allowed to bind to sections of peripheral lymph node tissue from an SIV-infected macaque with clinical AIDS. No detectable hybridization was obtained using the normal strand probe (Fig. 1*a*), whereas specific hybridization to large numbers of cells was obtained with the complementary strand probe (Fig. 1*b*). Thus, the protocol used was able to detect cells expressing SIV RNA and not double-stranded DNA within tissues. The same probes were also used with peripheral lymph node specimens from naïve, uninfected macaques, and no staining of cells was detected (data not shown).

In the first study, the non-isotopic ISH technique was applied to detect cells expressing SIV RNA in sections of spleen, thymus, peripheral and mesenteric lymph nodes of SIV-infected macaques at days 7, 14 and 28 p.i. In the tissues from macaques N17 and N18, sacrificed at day 7 p.i., large numbers of hybridized cells were detected in the mesenteric lymph node (Fig. 2*a*), whereas few hybridized cells were observed in the peripheral lymph node and spleen (Fig. 2*c*, *e*). Hybridization was identified in cells in all four tissues studied and thymus from macaques N19 and N20 at day 14 p.i. (Fig. 2*b*, *d*, *f*, *h*). At 28 days p.i., hybridization was observed at low levels in only one spleen and one mesenteric lymph node specimen from N21 and N22 (data not shown). In addition, there was a different distribution of hybridization-positive cells in lymph nodes on days 7 and 14 p.i. At day 7 p.i., hybridized cells were located in the paracortex (Fig. 2*a*, *c*, *e*), whereas, at 14 days p.i., hybridized cells were also observed in many of the germinal centres that were apparent in the lymph node at this time (Fig. 2*b*, *d*, *f*).

The observation of large numbers of hybridized cells in the mesenteric lymph node at day 7 p.i. led us to perform the second experiment. ISH was performed on sections of spleen, ileum and peripheral and mesenteric lymph nodes taken at autopsy from P213/P214 and P215/P216, killed humanely at 4 and 7 days p.i., respectively. No hybridized cells were detected in any sections taken at 4 days p.i. At 7 days p.i., hybridized cells were identified in all tissues examined (see Fig. 2*g* for example of ileum). Differences in the relative number of hybridized cells in the mesenteric lymph nodes compared with spleen and peripheral lymph nodes were not so marked for macaques P215 and P216 compared with N17 and N18. Nevertheless, a statistical analysis was performed of the number of hybridized cells in sections of spleen and peripheral and mesenteric lymph nodes of macaques N17, N18, P215 and N216, processed simultaneously. This indicated that there was a significantly higher density of cells that hybridized in the mesenteric lymph node compared with the spleen at 7 days p.i. (Table 2; Mann–Whitney U-test, $P < 0{\cdot}03$), but there was no significant difference between the number of hybridization-positive cells in mesenteric and peripheral lymph nodes at this time.

In sections of ileum, large numbers of hybridizing cells were identified 7 days p.i. (Fig. 2*g*). The cells were restricted primarily to Peyer's patches, but some were present in the lamina propria within intestinal villi.

Immunolabelling for SIV in lymphoid tissue

To complement ISH, immunolabelling techniques were developed using anti-SIV envelope (KK13; Kent *et al.*, 1991) and Nef (KK75; Arnold *et al.*, 1999) MAbs. Attempts to use

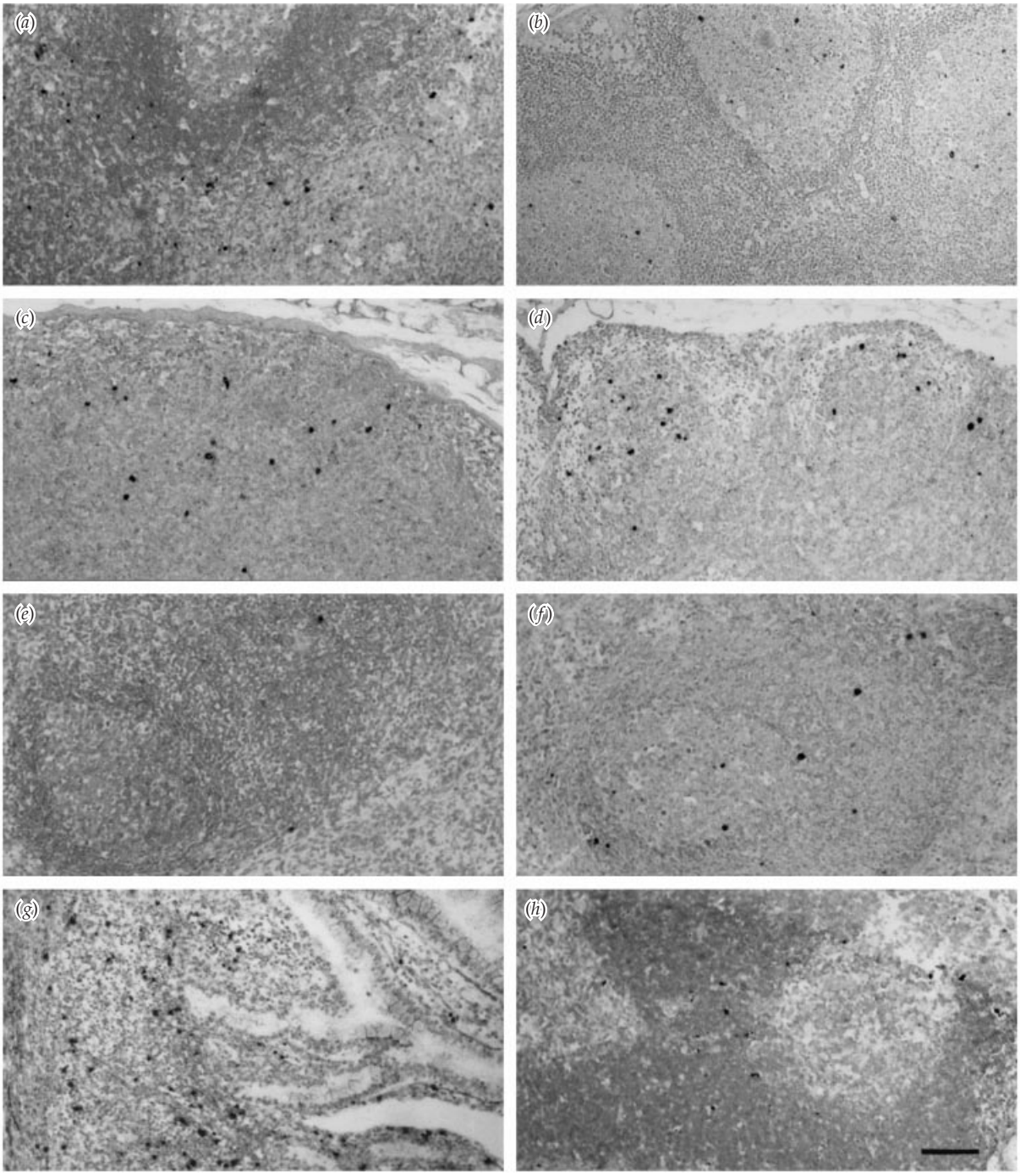

Fig. 2. Distribution of SIV-positive cells detected by ISH in the lymphoid tissues of cynomolgus macaques at 7 and 14 days after challenge. Cells that hybridized to a cocktail of three single-stranded, digoxigenin-labelled probes, complementary to regions of the SIV *gag*, *env* and *nef* genes, were stained black by the enzymatic conversion of the NBT/BCIP chromogenic substrate. Stained cells were identified in the mesenteric lymph node (*a*, *b*), peripheral lymph node (*c*, *d*), spleen (*e*, *f*), ileum (*g*) and thymus (*h*) removed from cynomolgus macaques at 7 (*a*, *c*, *e*, *g*) and 14 days (*b*, *d*, *f*, *h*) after inoculation with 10^3 $TCID_{50}$ SIVmacJ5. All micrographs are at the same magnification. Bar, 100 μm.

MAbs KK59 and KK61 (Kent *et al.,* 1991) to detect specifically SIV Gag protein were unsuccessful, as high levels of binding were observed with these antibodies on sections of lymph node processed from uninfected macaques (data not shown). No binding of MAbs KK13 and KK75 was observed on sections of lymph node from uninfected macaques.

Table 2. Quantification of positive cells by ISH in macaque tissue sections at day 7 after infection with SIVmacJ5

The numbers of positive cells are averages of two independent analyses of the same section. The area listed is the area of tissue sections over which analysis was performed. PLN, Peripheral lymph node; MLN, mesenteric lymph node.

Macaque	Tissue	No. of positive cells	Area (mm^2)	Density (positive cells per mm^2)
Experiment 1				
N17	MLN	235·0	88·90	2·64
	PLN	6·5	25·05	0·26
	Spleen	9·5	113·30	0·08
N18	MLN	124·0	44·86	2·76
	PLN	9·5	27·00	0·35
	Spleen	11·0	129·20	0·09
Experiment 2				
P215	MLN	135·0	44·10	3·06
	PLN	49·5	11·50	4·30
	Spleen	132·5	197·00	0·67
P216	MLN	341·0	25·86	13·19
	PLN	31·0	10·28	3·02
	Spleen	170·0	166·00	1·02

Table 3. Detection of SIVmacJ5 in the mesenteric lymph nodes of macaques by ISH or by immunolabelling with MAbs to SIV Nef and envelope proteins

Detection is scored as: −, no positive cells identified; +, small numbers of positive cells; ++, large numbers of positive cells; +++, large numbers of positive cells and diffuse staining of germinal centres. IML, Immunolabelling.

		Detection of SIV-positive cells by:		
Macaque	Time (days p.i.)	ISH	Anti-Nef IML	Anti-envelope IML
P213	4	−	−	−
P214	4	−	−	−
P215	7	+	+	+
P216	7	+	+	+
N17	7	+	+	+
N18	7	+	+	+
N19	14	+	+	+++
N20	14	+	+	+++
N21	28	−	+	++
N22	28	−	+	++

Parallel sections of mesenteric lymph node from each macaque were processed for ISH and immunolabelling with anti-envelope and anti-Nef MAbs. The results are presented in Table 3 and representative sections are shown in Fig. 3. No cells from material collected 4 days p.i. were labelled by either technique. At 7 days p.i., similar numbers of cells were labelled by all three methods. As expected from previous studies, SIV-positive cells were distributed in the paracortex (Fig. 3 *a*, *d*, *g*). At day 14 p.i., immunolabelling with anti-Nef MAbs and ISH visualized similar numbers of positive cells and, as expected, they were found within germinal centres as well as in the paracortex (Fig. 3 *b*, *e*). A very different distribution of labelling was obtained with the anti-envelope MAb (Fig. 3 *h*). More cells both within and between follicles were labelled with this antibody. In addition, a diffuse staining within the germinal centre of follicles was apparent (Fig. 3 *h*). At day 28 p.i., ISH did

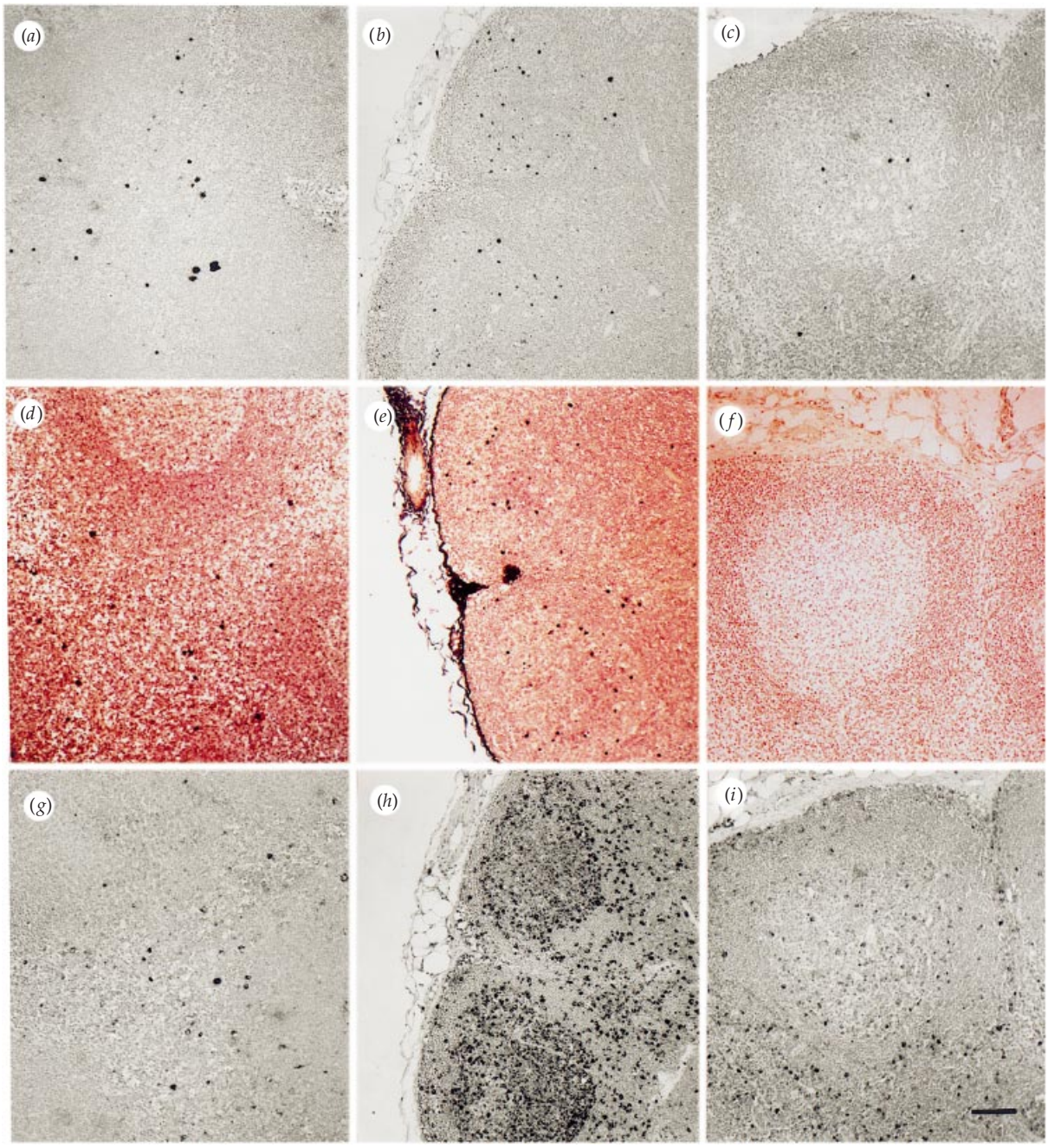

Fig. 3. Comparative distribution of SIV in lymphoid tissue detected by immunolabelling with anti-Nef or anti-envelope MAbs or by ISH. The presence of SIV was assessed in similar sections of mesenteric lymph nodes removed from cynomolgus macaques at 7 (*a*, *d*, *g*), 14 (*b*, *e*, *h*) or 28 (*c*, *f*, *i*) days after inoculation with 10^3 $TCID_{50}$ SIVmacJ5 by immunolabelling with an anti-Nef MAb, KK75 (*a–c*), ISH with a cocktail of three complementary-strand probes (*d–f*) or immunolabelling with an anti-envelope MAb, KK13 (*g–i*). All micrographs are at the same magnification. Bar, 100 μm.

not identify any SIV-positive cells (Fig. 3*f*), whereas immunolabelling with anti-Nef MAb detected small numbers of SIV-positive cells (Fig. 3*c*). Larger numbers of cells were identified as being SIV-positive by using the anti-envelope MAb. Nevertheless, the diffuse staining of the germinal centre was no longer apparent at this time (Fig. 3*i*).

Discussion

It is now widely believed that the initial interactions between HIV and the host are critical in determining the clinical outcome of infection. Characterization of the dissemination of the virus following infection is the first step in

understanding the pathogenesis of disease. In this study, we have characterized some aspects of virus dissemination that occur in the first 4 weeks following intravenous infection with the pathogenic virus SIVmacJ5 (Rud *et al.*, 1994). By killing humanely pairs of macaques at each time-point, we were able to study the distribution of virus in blood and peripheral and gut-associated lymphoid tissues by a number of different techniques. This study demonstrated that the gut-associated lymphoid tissue (mesenteric lymph nodes and Peyer's patches) contained large numbers of cells that actively expressed SIV transcripts by 1 week after intravenous inoculation of the virus. This marked concentration of actively replicating SIV-infected cells was not reflected by DNA PCR or virus co-culture assays to detect virus. Previous reports have used ISH to identify SIV-positive cells in gut-associated lymphoid tissue (Veazey *et al.*, 1998; Smit-McBride *et al.*, 1998). This is the first report where immunolabelling has been used to complement ISH and to characterize further the early pathology of SIV infection in cynomolgus macaques. When these results are considered with the knowledge that gut-associated lymphoid tissue contains the largest number of cells of any lymphoid tissue, one must conclude that gut-associated lymphoid tissue produces the majority of this virus during primary viraemia.

The seemingly discrepant results of virus co-culture, DNA PCR and ISH/immunolabelling reflect the related, but distinct, aspects of virus infection and replication that each technique identifies. It has been reported previously that analysis by virus co-culture and DNA PCR can yield unexpectedly distinct results, especially in the study of attenuated isolates of SIV (Almond *et al.*, 1995). The data obtained here at 4 weeks p.i. indicate clearly that cells that are not actively transcribing high levels of SIV RNA possess SIV DNA and can yield a productive infection if cultured with appropriate indicator cells.

A concern during the development and early application of the non-isotopic ISH assay was its sensitivity in comparison with assays that use radiolabelled probes. Certainly, our preliminary studies demonstrated that mixing all three probes (complementary to *gag*, *nef* and *env* genes) maximized sensitivity (unpublished observations). In addition, consecutive sections to those presented here were evaluated using ^{35}S-labelled SIV probes of high specific activity. No additional sections were identified as containing SIV-positive cells, nor was the frequency or distribution of positive cells observed to differ (T. A. Reinhart, personal communication). Furthermore, a close correspondence of the number and location of SIV-positive cells was obtained using ISH or immunolabelling with the anti-Nef MAb KK75 and the anti-envelope MAb KK13 at 1 week p.i. This would indicate that, at this time, immunohistochemistry and ISH were equally sensitive for detecting infected cells.

The development of immunolabelling protocols that are of equivalent sensitivity to ISH for the detection of cells that are actively replicating SIV adds a powerful new tool for the study of virus replication in tissues. Furthermore, the availability of two complementary MAbs against distinct SIV proteins has already proven valuable. MAb KK75 recognizes a linear peptide sequence in the SIV Nef protein (Arnold *et al.*, 1999). The Nef protein is present at high levels only in virus-infected cells. In contrast, MAb KK13 recognizes a conformation-dependent determinant in the SIV envelope protein, which is expressed not only in virus-infected cells but also on virions. The differential expression of these two SIV proteins is clear from the results obtained. In all sections stained with MAb KK75 at 1, 2 and 4 weeks after virus challenge, staining was localized over individual cells. In contrast, immunohistochemical staining with the anti-envelope MAb KK13 gave different patterns of staining depending upon the time after infection at which tissues were recovered. At 7 days p.i., the pattern with KK13 was very similar to that of KK75, indicating that a predominant location of envelope protein was on individual infected cells. However, a diffuse staining of germinal centres was observed at 14 days p.i. and a larger number of positively stained cells was detected at 28 days p.i. This would suggest that the major source of envelope protein detected at these later times is on virions that have been trapped on cells. A similar pattern of diffuse staining over germinal centres has been obtained when radiolabelled probes were used for ISH on lymphoid tissues recovered from macaques challenged with pathogenic isolates of SIV (Chakrabarti *et al.*, 1994*a*, *b*; Baskin *et al.*, 1995). In these previous reports, the pattern was ascribed to the detection of virions in the germinal centres. The distinct pattern of staining demonstrated in this study obtained by immunohistochemistry with MAbs against Nef, the viral regulatory protein, and envelope, the viral structural protein, provides confirmation of the previous tentative conclusions. If this diffuse staining is due to trapping of virions in the germinal centre, the loss of this diffuse staining by 4 weeks p.i. would suggest that neither macaque N21 nor N22 was likely to progress rapidly to disease (Chakrabarti *et al.*, 1994*a*).

In comparison with previous studies of the early pathology of SIV infection, the failure to detect any virus-positive cells by ISH at 4 weeks p.i. was unusual. However, previous comparative studies of SIVmacJ5 infection in rhesus and cynomolgus macaques have indicated that the primary viraemia in the blood is of lesser magnitude and shorter duration in the latter species. In addition, the cloned virus SIVmacJ5 is frequently less vigorous *in vivo* compared with uncloned SIVmac251 (11/88 pool; E. J. Stott and N. Almond, unpublished observations). Even in previous studies by others, a marked fall in the number of SIV-positive cells detected by ISH was observed between days 16 and 35 post-inoculation in rhesus macaques infected with the pathogenic clone SIVmac239 (Reimann *et al.*, 1994). However, by using ISH, we have detected recently hybridized cells in peripheral lymph nodes of J5-infected macaques at 16 weeks p.i. without patent progression onto clinical disease (N. Almond and D. Ferguson, unpublished).

Our observation that gut-associated lymphoid tissue, in particular mesenteric lymph nodes and Peyer's particle, was an early major site of virus replication following intravenous inoculation of virus contrasts with the observations of Stahl-Hennig *et al*. (1999), who characterized the primary viraemia of a closely related isolate of SIVmac251 after oral inoculation. Atraumatic administration of virus in the mouth was reported to result in very early replication of virus in the immediately local, and presumably activated, tonsillar lymphoid tissue before dissemination to distant gut-associated and peripheral lymphoid tissue (Stahl-Hennig *et al*., 1999). The distinct pattern of virus dissemination and replication perhaps reflects the lack of activated lymphoid tissue encountered by virus inoculated intravenously prior to the gut-associated lymphoid tissue, whereas the tonsils and related lymphoid tissue are designed to sample and deal with antigen and pathogens entering via the oral route.

The characterization of the early pathology of infection with SIVmacJ5 may be valuable in the development of an effective AIDS vaccine. The demonstration that, even after intravenous inoculation, gut-associated lymphoid tissue is a major site of initial virus replication needs to be borne in mind in the development of an effective vaccine strategy. In spite of the generation of strong serological and T cell responses to the virus in the periphery, recombinant subunit vaccines seldom confer the levels of protection obtained with live-attenuated virus vaccines. This may reflect an inability of subunit vaccines to generate effective responses in gut-associated lymphoid tissue. This study of the early pathology of SIV may account for the enhanced protection of subunit vaccines when they are targetted towards deep mesenteric lymph nodes (Lehner *et al*., 1996).

The development and application of a range of techniques to describe the distribution of a pathogenic isolate of SIV in the tissues of infected macaques will enable us to address further key questions. Of greatest importance is the identification of the first cells that are susceptible to infection with the inoculated virus and are moreover capable of disseminating the virus to further cells. The development of immunohistochemical reagents to detect SIV-infected cells will simplify the techniques of double labelling with antiviral antibodies and antibodies to cellular markers. Another intriguing question that may also be addressed with these techniques is the characterization of the changes in the early pathology of SIV infection *in vivo* that result from the disruption of selected regulatory genes. For example, the SIV clone C8 is attenuated through minor disruption of the *nef* gene. A comparison of the early pathology of C8 compared with J5 may provide insight into the function of the *nef* gene and its effect on the interaction between virus and host *in vivo*. This comparison may also help us to understand how *nef*-disrupted viruses confer potent vaccine protection against challenge with a broad range of pathogenic viruses (Almond *et al*., 1995; Almond & Stott, 1999). These studies of virus pathogenesis may therefore be of direct benefit in the development of a safe and effective AIDS vaccine.

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