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Human antibodies that neutralize primary human immunodeficiency virus type 1 *in vitro* do not provide protection in an *in vivo* model

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Recently, conflicting data have been published about the ability of antibodies which efficiently neutralize T cell-adapted human immunodeficiency virus type 1 (HIV-1) strains to neutralize primary HIV-1 strains *in vitro* and *in vivo*. Here we present data indicating that such antibodies fail to neutralize primary HIV-1 strains *in vivo*. To this end, a newly developed chimeric human-to-mouse model was used, in which several aspects of primary HIV-1 infection are mimicked. Poly- and monoclonal antibodies protected the grafted human cells, in a dose-dependent way, from infection with T cell-adapted HIV-1 in this system. A human monoclonal antibody specific for the CD4 binding domain that efficiently neutralizes HIV-1 IIIB *in vitro* did not protect the human graft from HIV-1 IIIB infection. None of the antibodies provided protection in the *in vivo* model against infection with primary HIV-1 strains, although they were able to neutralize these same strains *in vitro*.

Introduction

Studies aimed at the development of a vaccine against human immunodeficiency virus type 1 (HIV-1) have largely focused on the induction of antibodies which neutralize HIV-1 strains adapted to replication in T cell lines. It was shown that the neutralizing capacity of such antibodies was significantly lower or absent against primary HIV-1 strains, directly isolated in peripheral blood mononuclear cells (PBMC) from HIV-1-seropositive individuals (Moore *et al.*, 1995). The significance of these antibodies for *in vivo* protection and consequently the need to induce such antibodies by vaccination has been subject of fundamental debate (Schutten *et al.*, 1995; Moore, 1995). Evaluation of the potential of antibodies and antiviral compounds to interfere with the replication of primary HIV-1 strains *in vivo* has largely been hampered by the limited availability of suitable animal models. Besides the chimpanzee (*Pan troglodytes*) and the pigtail macaque (*Macaca nemestrina*), human–mouse chimeric models for HIV-1 infection have been described. These are based on the creation of a human type haematological environment by grafting human PBMC or haematopoietic tissue into SCID mice (Mosier *et al.*, 1991; Namikawa *et al.*, 1988); subsequently, the activated human graft is infected with HIV-1. In these models it was demonstrated that polyclonal hyperimmune globulins from seropositive donors (HIVIG), a monoclonal antibody (MAb) directed against the V3 loop on HIV-1 gp120 and 3′-azido-2′,3′-dideoxythymidine (AZT) inhibited the replication of T cell line-adapted (TCLA) HIV-1 strains (Safrit *et al.*, 1993; Shih *et al.*, 1991; McCune *et al.*, 1990). In infected individuals HIV-1 has been shown to replicate predominantly in activated macrophages and T lymphocytes resulting in high virus loads in lymphoid organs (Embretson *et al.*, 1993; Pantaleo *et al.*, 1993). With this in mind an alternative chimeric human-to-mouse model was developed in which human PBMC were grafted intraperitoneally (i.p.) into gamma-irradiated CBA/N mice. This results in an acute xenogenic-graft versus host disease (xeno-GvHD) providing a system in which human cells of both the monocytic and lymphocytic cell lineages become highly activated (Huppes *et al.*, 1992, 1993). In order to infect the grafted cells in a quiescent state, infection with HIV-1 was carried out within 1 h of grafting. Here we describe the replication kinetics of primary HIV-1 strains in this model. Furthermore, we assess the potential of different human HIV-1 neutralizing antibodies to interfere with the replication of TCLA and primary HIV-1 strains in grafted human cells.

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Methods

■ **Viruses and antibodies.** The antibody preparations used for passive immunization studies with the TCLA and primary HIV-1 strains were selected for a broad and high affinity for the HIV-1 strains used. They included: CD4 binding domain (bd)-specific human monoclonal antibody (HuMAb) GP13 (Schutten *et al.*, 1993); the HIV-1 IIIB V3 loop-specific mouse MAb F58H3 (Broliden *et al.*, 1992; Hinkula *et al.*, 1994); the HIV-1 V3 loop-specific HuMab 257-D (Gorny *et al.*, 1993); the gp41-specific HuMAb K14 (Teeuwsen *et al.*, 1990); and HIVIG (Prince *et al.*, 1991).

HIV-1 IIIB was kindly provided by the MRC AIDS Directed Programme and the virus stock was expanded using the CB15 $CD4^+$ T cell line (Gallo *et al.*, 1983; Nick *et al.*, 1993). The primary HIV-1 molecular clones 320.2A.1.2 [SI (syncytium inducing/non-macrophage tropic)] and 320.2A.2.1 [NSI (non-syncytium inducing/macrophage tropic)], and the primary HIV-1 strains ACH 172.BA-L (NSI) and ACH 168.10 (SI), were provided by H. Schuitemaker from the Central Laboratory for the Blood Transfusion Service in Amsterdam. Their *in vitro* passage history has been described previously (Schuitemaker *et al.*, 1992*a*, *b*, 1993). The full-length infectious molecular clones HIV-1 320.2A.1.2 (SI) and HIV-1 320.2A.2.1 were transfected into the $CD4^-$ cell line COS and cell-free supernatants were obtained after 3 days. A high titred virus stock from HIV-1 320.2A.1.2 (SI) was made within 2 weeks of primary infection using the CB15 cell line. During this short single passage it is unlikely that the genotype and phenotype of the HIV-1 molecular clone changed significantly (Back *et al.*, 1993; Gartner & Popovic, 1990). Since HIV-1 320.2A.2.1 (NSI) does not replicate to high titres in phytohaemagglutinin (PHA)-stimulated PBMC and immortalized T cell lines, xeno-GvHD mice were infected with HIV-1 320.2A.2.1 (NSI) and after 2 weeks peritoneal tissues from these mice were cultured for another 6 days to produce high titre virus stocks. Virus stocks from ACH 168.10 (SI) and ACH 172.BA-L were produced using PHA-stimulated human PBMC according to established procedures (Schuitemaker *et al.*, 1992*a*).

■ **Xeno-GvHD mice.** Xeno-GvHD mice were generated as previously described by Huppes *et al.* (1992). In brief, 4 to 5-week-old CBA/N/Rij mice (Harlan CPB, Zeist, The Netherlands), bred and kept under specific pathogen-free conditions, were conditioned by total body irradiation (9 Gy) with haematological support of 5×10^5 syngeneic bone marrow cells intravenously. For grafting 2×10^7/g bodyweight human PBMC isolated on a Ficoll gradient were used; this has been shown to induce an acute GvHD in 100% of cases (Huppes *et al.*, 1992, 1993). Mice within an experiment received PBMC from one individual only. The data plotted in Figs 2 and 3 represent the results of one experiment with data measurements at a single time-point. All experiments were performed in duplicate with similar results. Each virus–antibody combination was therefore tested with PBMC from at least six different donors.

■ **Virus load.** The virus load of the human lymphocytes isolated from the xeno-GvHD mice infected with the different HIV-1 strains was determined with an infectious centre test (ICT). Human lymphocytes from peritoneal lavages of each mouse were counted and titrated individually in duplicate starting at $2{\cdot}5 \times 10^6$ per well in 96-well round bottom plates. Human PBMC that had been prestimulated with PHA for 3 days were also added at a concentration of 5×10^4 per well. Cells were cultured for 1 week in RPMI 1640 (GIBCO BRL), 10% heat inactivated fetal bovine serum (Hyclone Laboratories Inc.), penicillin (100 U/ml), streptomycin (100 μg/ml) and 50 units of recombinant human IL-2/ml. (Proleukin, EuroCetus). After 7 days, 50 μl of supernatant per well was tested in a p24 antigen ELISA (V5 p24 antigen ELISA kit; a kind gift of Organon Teknika, Boxtel, The Netherlands) according to the manufacturer's instructions. The minimum number of cells from the peritoneal lavage from each individual mouse that was required to detect viral antigen in more than 50% of the wells was taken as a measure of viral load. Both peritoneal tissue and spleen were also cultured using the same culture medium for detection of productively infected cells.

■ **Immunohistochemistry and *in situ* hybridization.** CD45 (human leukocyte marker) and CD68 (human macrophage marker) immunohistochemistry was performed as described previously (Tenner-Racz *et al.*, 1994). Sequential series of 4 μm sections were stained for HIV-1 in combination with CD45 and CD68. For detection of productively infected cells by *in situ* hybridization, a ^{35}S-labelled antisense RNA probe of HIV-1 and a ^{35}S-labelled sense RNA probe as negative control (Lofstrand Labs) were used. Sections (4 μm) of formalin-fixed paraffin-embedded tissues were digested with Proteinase K (Sigma) at 37 °C for 15 min. The tissue sections were subsequently acetylated in 0·25% acetic anhydride–0·1% trietholamine pH 8·0 at room temperature for 5 min and subsequently prehybridized with 50% formamide, 0·5 M-NaCl, 10 mM-Tris–HCl pH 7·4, 1 mM-EDTA, 0·02% Ficoll–polyvinyl pyrrolidone–BSA and 2 mg/ml tRNA for 2 h at 45 °C. Radiolabelled probe (2×10^6 d.p.m./μl) in prehybridization mixture + 10% dextran sulphate was added to the slides and incubated overnight at 45 °C. After extensive washing and RNAse digestion (Boehringer Mannheim) tissue sections were dehydrated and incubated with Kodak NTB-2 emulsion and developed in Kodak D-19 developer; the sections were then counter-stained with haemalum and mounted. Also, photographs were taken of the *in situ* hybridization slides using excitation at one specific wavelength to identify the silver grains showing HIV-1-specific *in situ* hybridization.

Results

HIV-1 infection in xeno-GvHD mice

Xeno-GvHD mice were infected i.p. with 30 xeno-GvHD mice infectious doses 50% ($XeID_{50}$) of primary and TCLA HIV-1 strains with SI and NSI phenotypes. These included the TCLA HIV-1 strain IIIB and the primary HIV-1 strains 320.2A.1.2 (SI), 320.2A.2.1 (NSI), ACH.172.BA-L (NSI) and ACH 168.10 (SI). Directly after development of acute xeno-GvHD, combined *in situ* hybridization and immunohistochemistry studies were carried out, using an HIV-1 probe and CD45 and CD68 specific conjugates, on peritoneal tissues and lymphoid organs of mice infected with the primary HIV-1 strains. Acute xeno-GvHD consistently started between 6 to 14 days after grafting, depending on the numbers of human PBMC grafted. The HIV-1-specific staining showed a significant increase between days 6 and 12 after grafting (not shown). Fig. 1(*a–c*) shows a sequential series of sections from part of the diaphragm stained for the macrophage marker CD68 and HIV-1. Large numbers of human macrophages were present in the peritoneal tissues of these mice. In mice infected with the NSI, HIV-1 strains, like HIV-1 320.2A.2.1 (Schuitemaker *et al.*, 1992*a*), HIV-1-specific staining was found mainly in these areas. HIV-1-specific staining of T cell-rich areas was also observed, although to a lesser extent since HIV-1 320.2A.2.1 also infects $CD4^+$ T cells. In mice infected with SI HIV-1 strains like 320.2A.1.2 and 168.10, which do not infect monocyte-derived macrophages *in vitro* (Schuitemaker *et al.*, 1992*a*), HIV-1-specific staining was significantly lower in the

macrophage-rich areas (Fig. 1*d–f*). However, in the lymphoid organs of these mice, as in the periarteriolar lymphoid sheath shown in Fig. 1(*g–i*) where predominantly human T cells and hardly any human macrophages were present, abundant HIV-1 staining could also be demonstrated. The number of productively infected cells per field at the same magnification was about ten times higher in these T cell-rich areas compared to tissues in the peritoneal cavity. The numbers of HIV-1-infected human cells in peritoneal lavages of those mice whose tissues were used for immunohistochemistry and *in situ* hybridization were determined in the ICT. For the primary HIV-1 strains, ratios between infected and non-infected cells increased from about 10^{-4} at day 6 to more than $10^{-1.5}$ at day 12 after grafting. This proved not to be related to the capacity of the virus to induce syncytia *in vitro*. HIV-1 IIIB infection in the xeno-GvHD model proved to be self-limiting since the ratio of infected and non-infected cells in the peritoneal lavage cells was 10^{-4} at day 6 and diminished to an undetectable level at day 11 (Fig. 2*a*, *b*). Infectious HIV-1 could not be detected at day 11 in spleen or peritoneal tissues of mice infected with HIV-1 IIIB.

Passive immunization studies with HIV-1 IIIB

Intraperitoneal administration of 20 mg/kg poly- or monoclonal antibody preparations, 1 h prior to grafting of the PBMC, followed by infection with 30 $XeID_{50}$ HIV-1 IIIB, resulted in different patterns of interference with virus replication (Fig. 2*a*). Administration of the CD4 bd-specific HuMAb GP13, which neutralizes HIV-1 IIIB *in vitro* [inhibitory concentration 90% (IC_{90}) = 2 μg/ml) (Back *et al.*, 1994)] had no effect on the virus load in human cells at day 6 in the peritoneal lavages. Administration of the V3 loop-specific mouse MAb F58H3 either alone or in the presence of HuMAb GP13 (both administered at 20 mg/kg), and administration of HIVIG, resulted in the absence of detectable HIV-1 IIIB infected cells in peritoneal lavages, peritoneal tissues and spleen. The minimum concentration of mouse MAb F58H3 that interfered with the numbers of detectable HIV-1 IIIB infected cells was determined by titrating the antibody *in vivo*. Between 10 and 20 mg/kg was needed to abolish the presence of HIV-1 IIIB-infected cells (Fig. 2*c*). At day 11, HIV-1 IIIB was detected by ICT in the peritoneal lavage cells from xeno-GvHD which had received the CD4 bd-specific HuMAb GP13. This is surprising since peritoneal lavage cells, spleen and peritoneal tissue collected at day 11 from xeno-GvHD mice that had received identical numbers of PBMC from the same blood donor and the same dose of HIV-1 IIIB were negative in the ICT (Fig. 2*b*).

Passive immunization studies with primary HIV-1

In contrast, i.p. administration of 20 mg/kg of the HuMAbs or HIVIG, prior to infection of the human PBMC graft with 30 $XeID_{50}$ of the primary HIV-1 strains, did not result in a significant reduction in the virus load in any of the antibody–primary HIV-1 strain combinations tested (Fig. 3*a–d*). The antibody preparations used in these assays included the following.

CD4 bd-specific HuMAb GP13, which neutralizes TCLA HIV-1 strains *in vitro* (Back *et al.*, 1994).

V3 loop-specific HuMAb 257-D, which neutralizes the primary HIV-1 strain 320.2A.1.2 *in vitro* with an IC_{50} of 10 μg/ml and an IC_{90} of 35 μg/ml.

A combination of equal amounts of these two antibodies, which neutralized HIV-1 320.2A.1.2 (SI) *in vitro* better than HuMAb 257-D alone, with an IC_{50} of 6 μg/ml and an IC_{90} of 20 μg/ml.

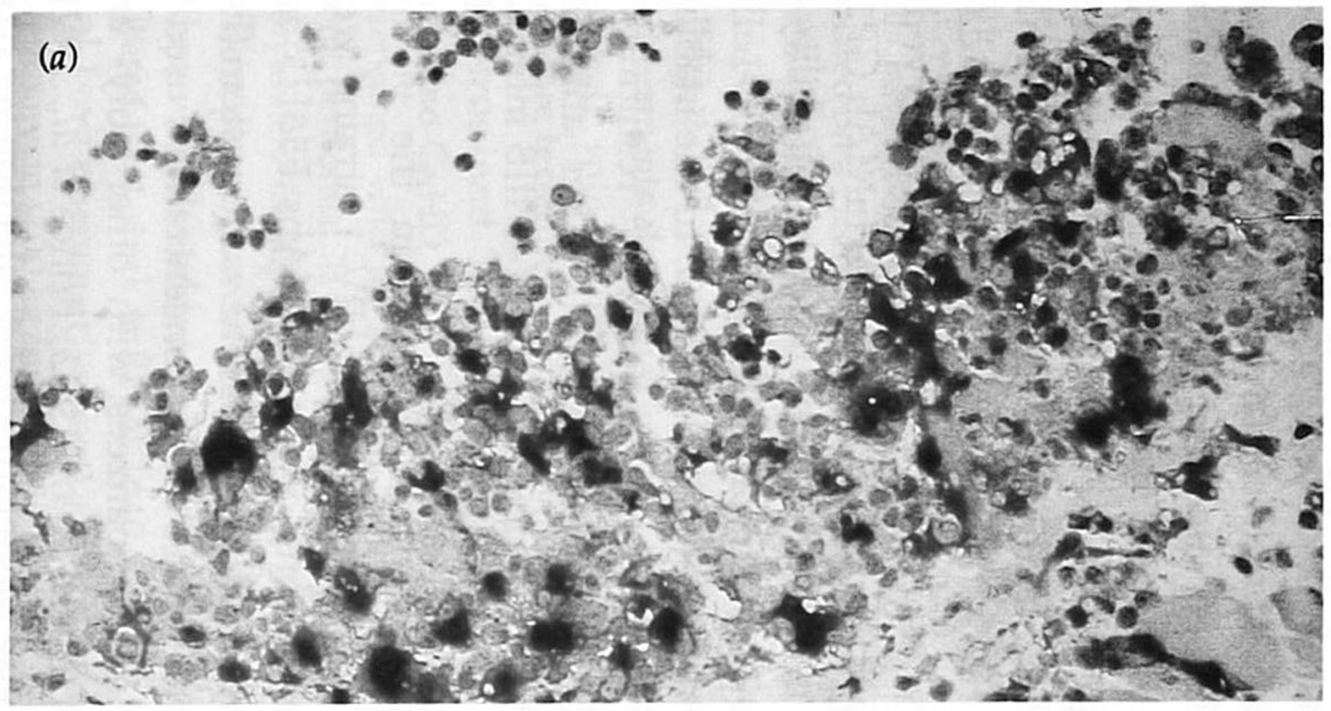
Polyclonal HIVIG preparation, which had previously been shown to neutralize HIV-1 IIIB *in vitro* and *in vivo* (Prince *et al.*, 1991) (Fig. 1) and primary HIV-1 strains *in vitro* (H. Schuitemaker, personal communication).

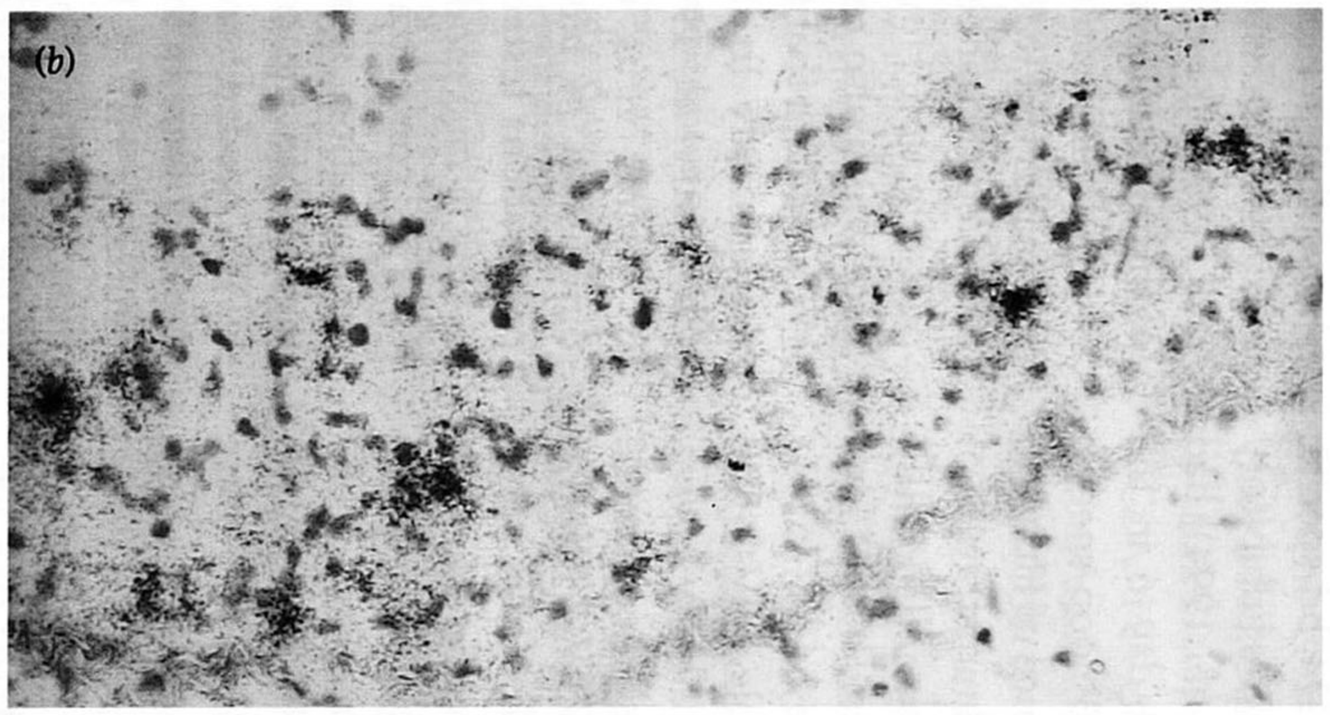
Discussion

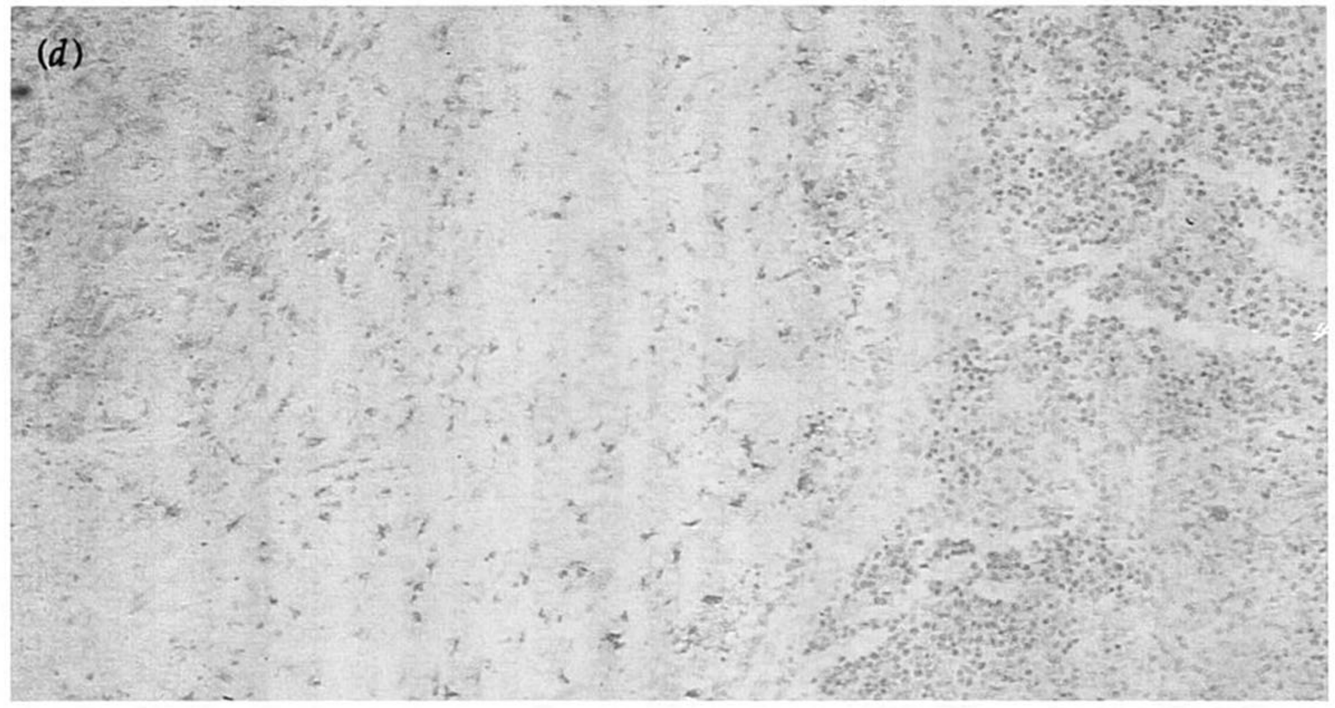
Recently, conflicting data have been published about the capacity of antibodies which efficiently neutralize T cell-adapted HIV-1 strains to neutralize primary HIV-1 strains *in vitro* and *in vivo*. Here, we present data indicating that such antibodies fail to neutralize primary HIV-1 strains *in vivo*.

In the HuPBL-SCID and HuCBL-SCID models it has been shown that in more than 50% of the grafts HIV-1 IIIB fails to establish a persistent infection (Reinhardt *et al.*, 1994; Mosier *et al.*, 1991). It has been speculated that the self-limiting nature of this infection is due to the relatively high cytopathogenicity of HIV-1 IIIB resulting in the elimination of all permissive cells in infected foci (Mosier & Sieburg, 1995). In our model we also observed clearance of HIV-1 IIIB infection as early as 11 days post-infection. However, a persistent infection was observed in mice which also received the CD4 bd-specific HuMAb GP13. We speculate that this is due, on the one hand, to the ability of the antibody to inhibit syncytium induction of HIV-1 IIIB (Schutten *et al.*, 1993), and, on the other hand, to the inability to inhibit virus entry (Fig. 1*a*), collectively resulting in diminished *in vivo* cytopathogenicity. All other antibody preparations tested efficiently neutalized HIV-1 IIIB infection in this *in vivo* model.

The concentrations of antibodies used in the *in vivo* neutralization assays with the primary HIV-1 strains were higher than the concentrations of mouse MAbs F58H3 and HIVIG needed to neutralize HIV-1 IIIB in the same system (Fig. 1). They were in the same range as the concentrations used to neutralize HIV-1 IIIB in chimpanzees with other poly- and monoclonal antibodies (Emini *et al.*, 1992; Prince *et al.*, 1991). In the xeno-GvHD mouse model for HIV-1 infection we have recently also tested negatively charged succinylated human serum albumins (Suc-HSA), which neutralize TCLA and







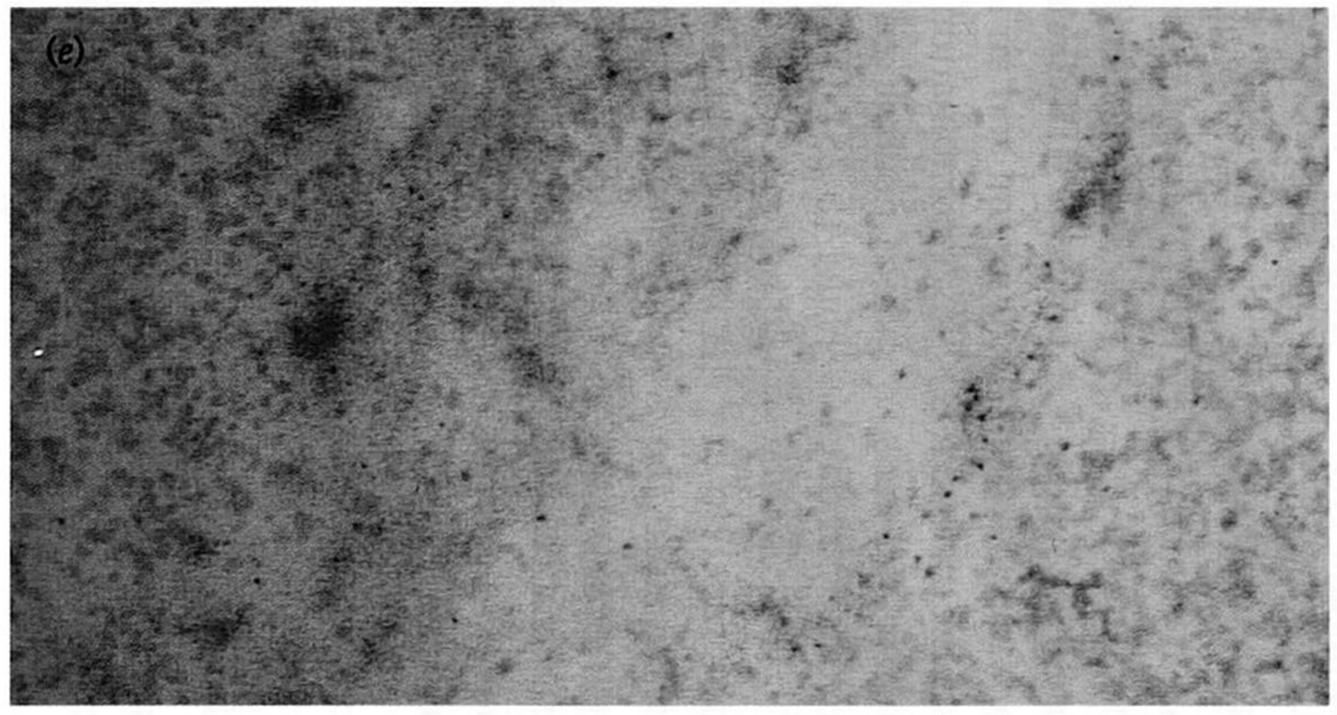


Fig. 1 (*a–f*). For legend see opposite.

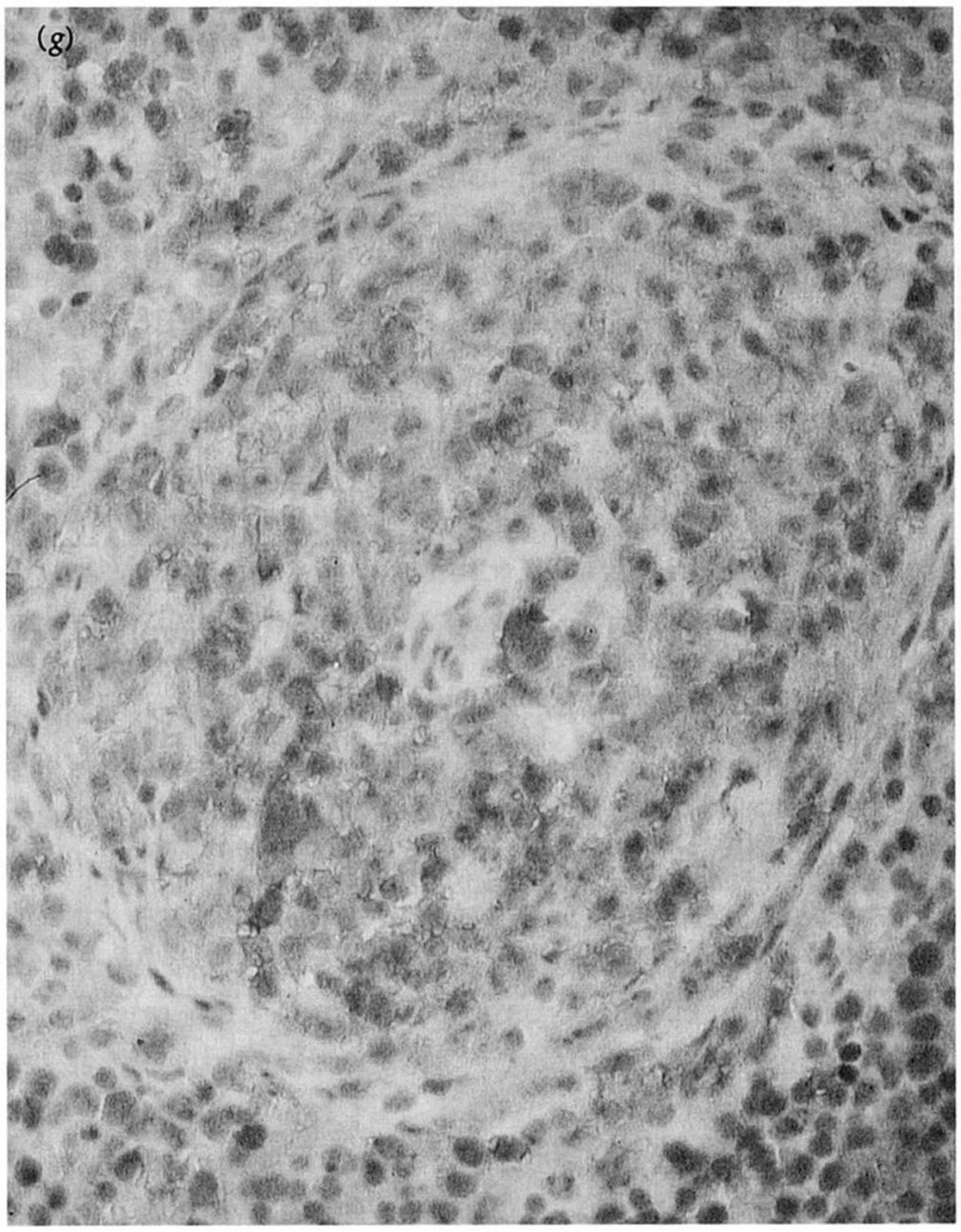
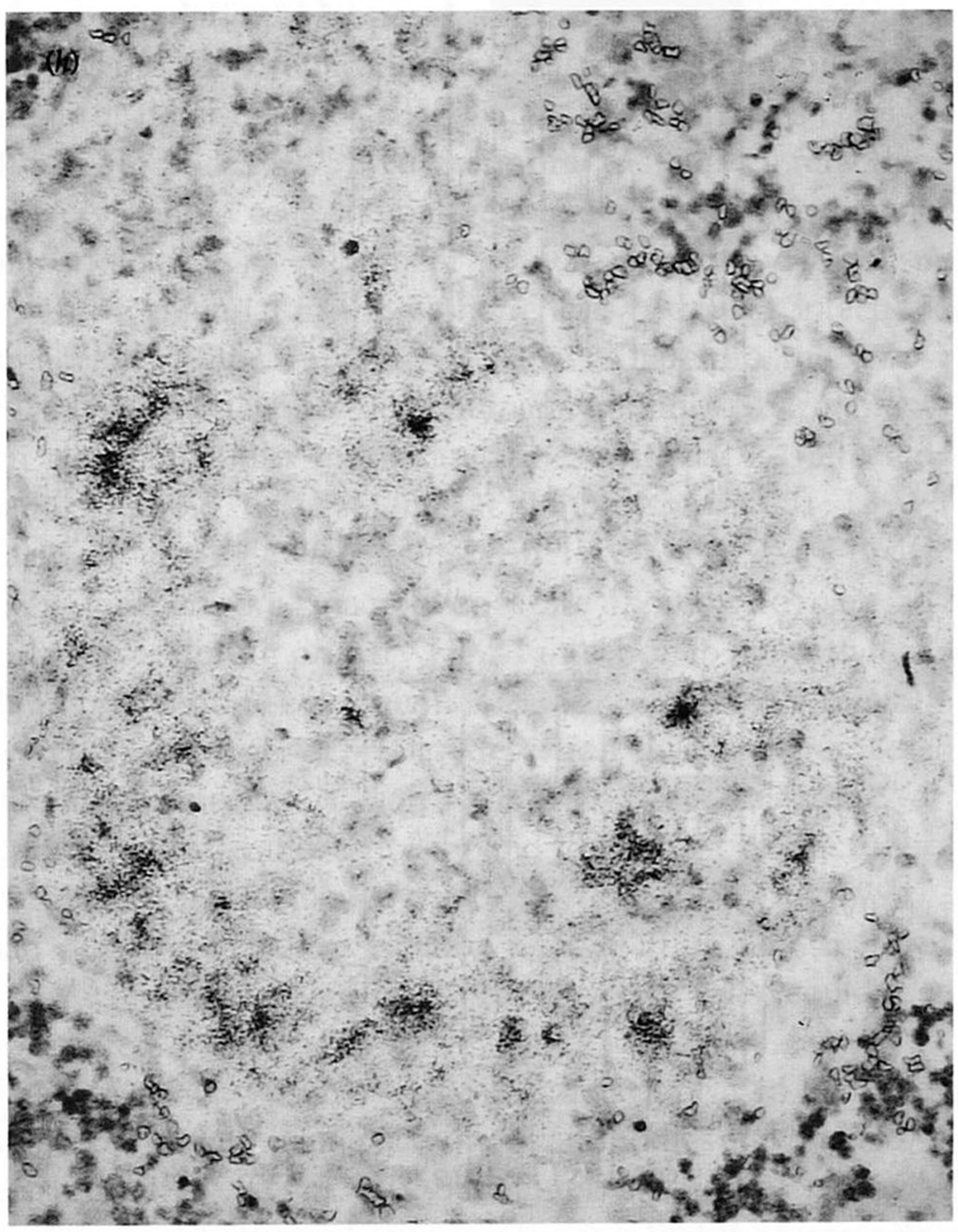
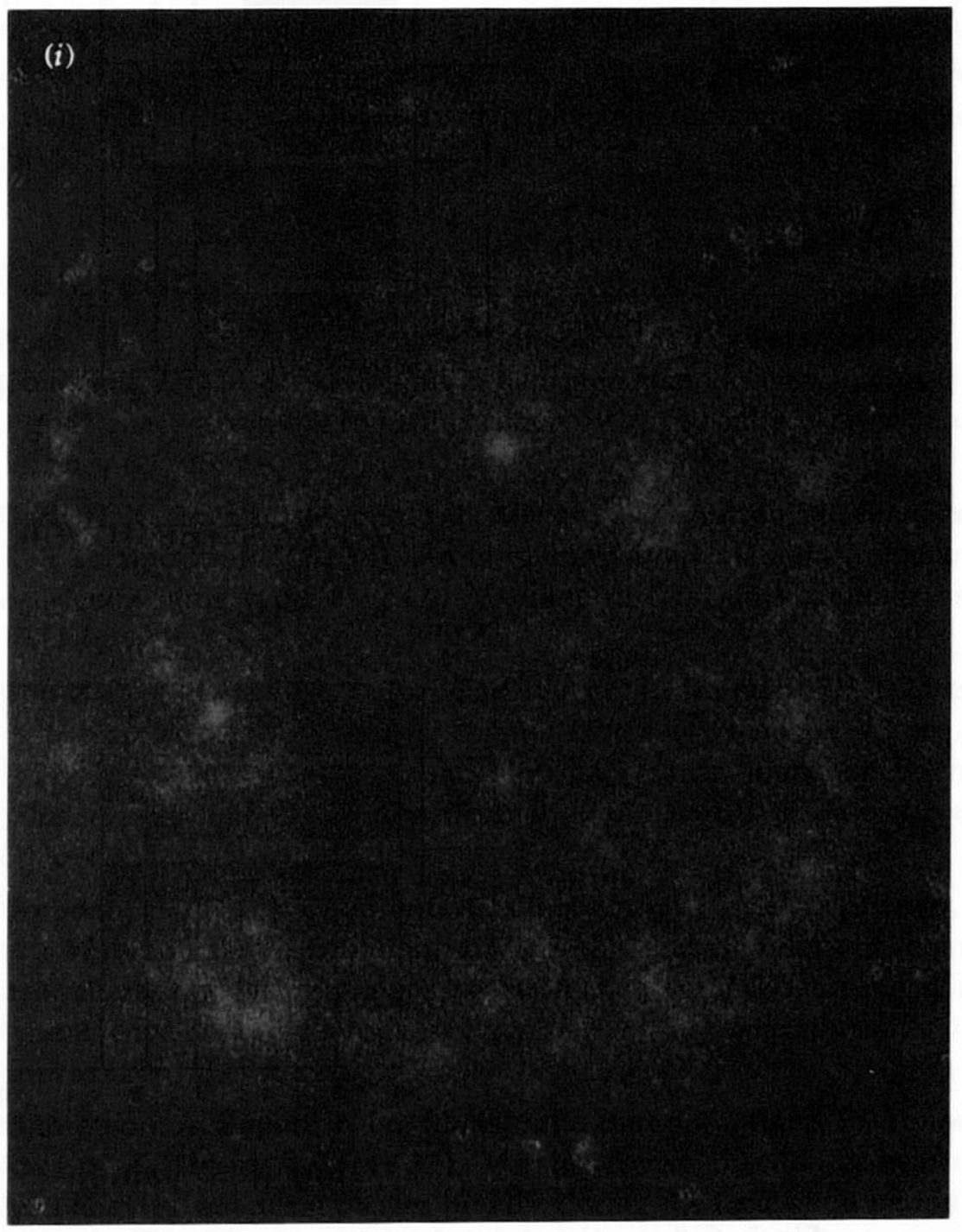


Fig. 1. HIV-1 *in situ* hybridization, CD45 and CD68 immunohistochemistry of spleen and peritoneal tissue from xeno-GvHD mice infected with either 320.2A.1.2 (SI, non-macrophage tropic) or 320.2A.2.1 (NSI, macrophage tropic). (*a–c*) Sections from the diaphragm of xeno-GvHD mice infected with HIV-1 320.2A.2.1 and stained for CD68 (*a*) or HIV-1 RNA (*b*) and epifluorescence of the same HIV-1 RNA *in situ* hybridized section (*c*) (magnification 220 ×). (*d–f*) Sections from the spleen (at the right-hand side of the figure) and pancreas (at the left-hand side of the figure) infected with HIV-1 ACH 168.10 and stained for CD68 (*d*) or HIV-1 RNA (*e*), and epifluorescence of the same HIV-1 RNA *in situ* hybridized section (*f*) (magnification 44 ×). (*g–i*) Sections from the spleen of HIV-1 320.2A.1.2-infected xeno-GvHD mice, stained for CD45 (*g*) or HIV-1 RNA (*h*), and epifluorescence of the same HIV-1 RNA *in situ* hybridized section (*i*) (magnification 430 ×).





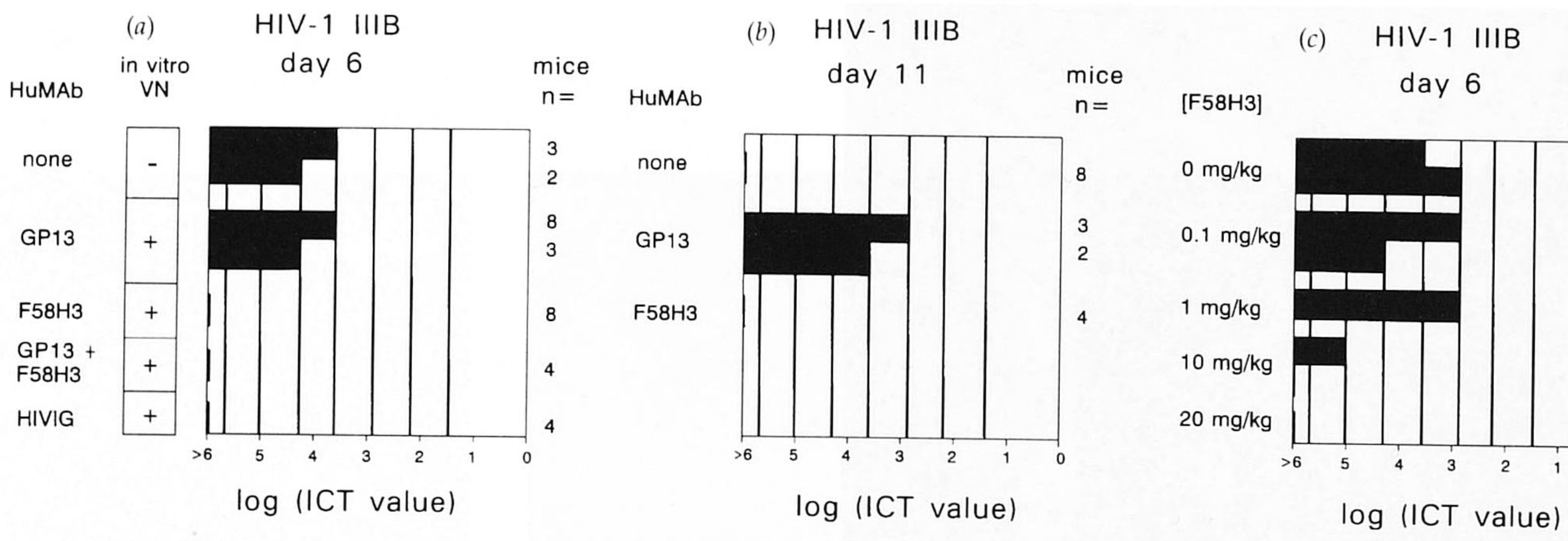


Fig. 2. Cell-associated virus load determined by ICT in human cells from peritoneal lavages of xeno-GvHD mice at the day xeno-GvHD was observed in mice infected with 30 $XeID_{50}$ HIV-1 IIIB. Prior to infection, MAbs or HIVIG were administered i.p. at 20 mg/kg. In the antibody mixtures (V3 specific MAb + CD4 bd specific HuMAb) 20 mg/kg of each antibody was given. In (c) different concentrations of the V3 specific MAb F58H3 were tested as indicated on the left-hand side of the plot. The number of mice used per group is indicated on the right-hand side of each plot (n). On the horizontal axis the logarithm of the numbers of cells needed to yield positive cultures in the ICT are given. Solid grid lines indicate the actual dilutions made (5-fold dilution steps starting at 5×10^5 cells). Where $> 10^6$ cells is indicated, none of the mouse tissues tested in culture (peritoneal tissue, spleen, ascitic fluid cells) proved to be positive for HIV-1 p24 after 7 days of culture.

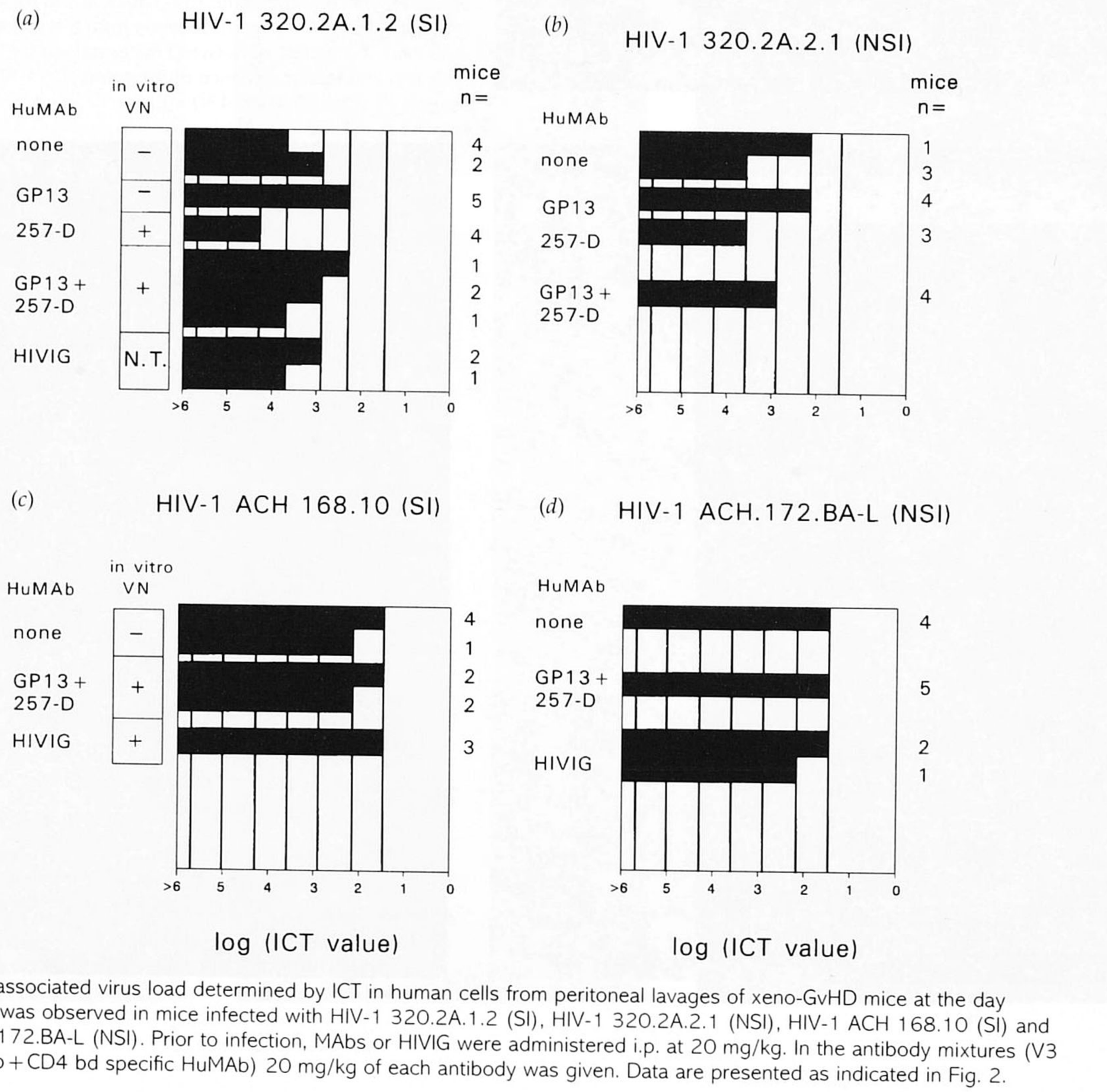


Fig. 3. Cell-associated virus load determined by ICT in human cells from peritoneal lavages of xeno-GvHD mice at the day xeno-GvHD was observed in mice infected with HIV-1 320.2A.1.2 (SI), HIV-1 320.2A.2.1 (NSI), HIV-1 ACH 168.10 (SI) and HIV-1 ACH 172.BA-L (NSI). Prior to infection, MAbs or HIVIG were administered i.p. at 20 mg/kg. In the antibody mixtures (V3 specific MAb + CD4 bd specific HuMAb) 20 mg/kg of each antibody was given. Data are presented as indicated in Fig. 2.

primary HIV-1 strains by binding to the V3 loop of gp120 (Jansen *et al.*, 1993). The concentration of Suc-HSA needed to neutralize HIV-1 IIIB in the xeno-GvHD mouse system was approximately the same on a molar basis as that of the V3 loop-specific mouse MAb F58H3 (Fig. 1) (Kuipers *et al.*, 1996). Preliminary data, however, showed that a 100-fold higher concentration failed to provide neutralizing activity towards primary HIV-1 strains. This concentration is more than 50 times the *in vitro* IC_{50} against HIV-1 320.2A.1.2 used in this study (unpublished results). Therefore, we conclude that the inability of the V3 loop-specific antibodies to neutralize primary HIV-1 strains *in vivo* cannot be attributed to the use of insufficiently high concentrations of these compounds in our *in vivo* model.

These data extend *in vitro* observations showing that primary HIV-1 strains are more resistant to virus neutralization in human PBMC than TCLA strains (Moore *et al.*, 1995; Bou-Habib *et al.*, 1994; Schutten *et al.*, 1995). The relevance of the *in vivo* data generated with primary HIV-1 strains in the xeno-GvHD model in which no protection was found, as compared to the *in vitro* data showing limited neutralization of these strains, is determined by the similarities between HIV-1 infection in humans and HIV-1 infection in this model. Like HIV-1-infected human lymphoid tissues, the model provides a substrate of highly activated cells of the monocytic and T lymphocytic lineages, of which the latter are skewed toward a $CD4^+$ subpopulation (Huppes *et al.*, 1992, 1994). This allows high levels of HIV-1 replication, a feature that is thought to be essential for persistence of HIV-1 infection in humans (Wei *et al.*, 1995; Ho *et al.*, 1995). Furthermore, the close interaction between lymphoid cells and antigen-presenting cells, which is a hallmark of the development of acute xeno-GvHD, also seems to be essential in the pathogenesis of HIV-1 infection (Gartner *et al.*, 1986; Huppes *et al.*, 1992). Therefore, the xeno-GvHD HIV-1 model provides a human lymphoid environment which clearly exhibits more similarities with HIV-1-infected human lymphoid tissues (Fig. 2) (Embretson *et al.*, 1993; Pantaleo *et al.*, 1993) than *in vitro* HIV-1-infected PBMC cultures. Furthermore, macrophages are thought to play an important role during primary HIV-1 infection in humans. The *in situ* hybridization experiments with HIV-1 320.2A.2.1 (NSI) showed that at the site of primary infection, macrophages were productively infected. In order to mimic primary HIV-1 infection in humans more closely and in contrast to the other chimeric models (Namikawa *et al.*, 1988; Mosier *et al.*, 1991), human cells were challenged soon after grafting, long before the characteristic activation of the grafted cells could be demonstrated.

The reason why primary HIV-1 strains appear to be more resistant to the neutralizing activity of antibodies and Suc-HSA *in vivo* than their TCLA HIV-1 counterparts is not clear. A major difference between both virus types is that the former do not shed gp120 as readily (Moore *et al.*, 1992; Groenink *et al.*, 1995). It may be speculated that after binding of the virus to the receptor, V3-specific antibodies may at least temporarily prevent the process of fusion between the virus- and cellular-membranes. This leaves the TCLA viruses in particular vulnerable to gp120 shedding without being able to penetrate the cell membrane, which eventually results in more effective neutralization of TCLA HIV-1 strains. Finally, it should be realized that other mechanisms of antibody-mediated enhancement of infectivity have been described. At least one of these may also be dependent on the phenotype of the virus and the cell type involved (Schutten *et al.*, 1995). The lack of neutralizing activity found in the xeno-GvHD mouse model with the antibody preparations tested may therefore also partly be due to the interference of enhancing antibody activity.

Taken together, our data show that primary HIV-1 strains cannot easily be neutralized in an *in vivo* system that closely mimics HIV-1 infection in humans. Since induction of virus-neutralizing antibodies is still one of the major targets in the development of candidate HIV-1 vaccines, we propose to use for their evaluation assay systems like the xeno-GvHD mouse model for HIV-1 infection.

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