humidity for 12 to 14 days [1 to 2 days longer than in (*15*)] or until no additional flies emerged.

The current optimal procedures produce overall survivals that constitute effective and practical cryobiological preservation of the Oregon R strain. Although it remains to be determined whether they will also be effective in preserving embryos from mutant lines, the major developmental processes are completed by stage 16, and consequently we expect that most laboratory strains will tolerate these vitrification procedures as well as Oregon R does. An advantage of defining the optimum developmental time for cryopreservation in relation to the time at which the ratio of stages 14 to 15 is 1:1 is that it will automatically compensate for any strain-to-strain differences in development rate. The approach may also be applicable to the cryopreservation of embryos from other diptera like the housefly or mosquito, which have developmental rates very different from that of *Drosophila* (*20*).

We believe our findings have more general implications for cryobiology. The optimal developmental stages being frozen are probably the most complex systems that have been cryobiologically preserved. The embryos are highly differentiated into tissues and organs including muscle and nerve, which indicates that differentiated multicellularity is not a barrier to cryopreservation per se. The findings also represent perhaps the first case in which vitrification procedures are required to obtain survival. From the mechanistic point of view there remains the question of why *Drosophila* survival is so critically dependent on developmental stage. Older embryos may vitrify more readily than younger embryos, or possibly they tolerate the presence of ethylene glycol or small amounts of ice better than younger embryos, perhaps because the critical steps of dorsal closure and head involution are completed. The answers could be important in determining the extent to which the general strategies described prove applicable to other non-mammalian eggs and embryos, most of which have not been successfully cryobiologically preserved.

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21. Permeabilization was effected by successive exposure of embryos on 25-mm Nuclepore or Poretics polycarbonate filters to 2.5% hypochlorite to remove the chorion, a thorough water wash, isopropanol treatment, air-drying for 2 min to remove the isopropanol, and a mixture of 0.3% 1-butanol in *n*-heptane for 90 s, followed by a brief chase with pure heptane. Details are given in (*7*) and (*15*). The duration of the air-drying and the butanol/heptane steps are critical. The filters were rinsed several times in D-20 and then incubated 45 min at 24° to 25°C. The percentage of permeabilized embryos was defined as the percentage that stained red or dark pink after a 5-min exposure at 23°C to 0.1% rhodamine B in D-20. [The leftmost and rightmost points of the dashed curve are from (*15*).] The embryos on the filters were then exposed to 2 M ethylene glycol at room temperature (23°C) for 30 min and then to 8.5 M ethylene glycol plus 10% (w/v) polyvinyl pyrrolidone (Plasdone C-30) for 4.5 to 5.5 min at 5°C. The next step was to abruptly plunge the filters into a mixture of solid and liquid nitrogen (nitrogen slush) at approximately −205°C and then 10 to 30 s later to abruptly plunge the filters into 0.75 M sucrose in D-20 at 23°C, hold them in that solution for 2 min, and then transfer them into D-20. Details of and reasons for these various steps are given in (*15*).

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Cellular Proteins Bound to Immunodeficiency Viruses: Implications for Pathogenesis and Vaccines

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Cellular proteins associated with immunodeficiency viruses were identified by determination of the amino acid sequence of the proteins and peptides present in sucrose density gradient–purified human immunodeficiency virus (HIV)–1, HIV-2, and simian immunodeficiency virus (SIV). β2 microglobulin (β_2m) and the α and β chains of human lymphocyte antigen (HLA) DR were present in virus preparations at one-fifth the concentration of Gag on a molar basis. Antisera to HLA DR, β_2m, as well as HLA class I precipitated intact viral particles, suggesting that these cellular proteins were physically associated with the surface of the virus. Antisera to class I, β_2m, and HLA DR also inhibited infection of cultured cells by both HIV-1 and SIV. The specific, selective association of these cellular proteins in a physiologically relevant manner has major implications for our understanding of the infection process and the pathogenesis of immunodeficiency viruses and should be considered in the design of vaccines.

Studies with subunit vaccines have shown that immunizations with viral envelope antigens alone are sufficient to elicit protective immunity against SIV or HIV (*1*). The recent observation (*2*) that macaques immunized with uninfected human cells were protected against challenge with SIV grown in human cells raised the possibility that immune responses to cellular antigens might also be involved in protection. However, the putative cellular antigens that may be stimulating the protective response have not been identified, and the mechanism of protection is unclear.

To identify the specific cellular antigens associated with immunodeficiency viruses, we purified and sequenced proteins from preparations of HIV-1, HIV-2, and SIV.

Viruses were propagated in H9 cells, concentrated by sucrose density gradient ultracentrifugation, and centrifuged to pellet the virus (*3*). Proteins from purified virus were separated by reversed-phase high-pressure liquid chromatography (rp-HPLC) as described (*4*) (Fig. 1). The predominant cellular proteins found in purified HIV-1, HIV-2, and SIV were β2 microglobulin (β_2m), human lymphocyte antigen (HLA) DR (α and β chains), actin, and ubiquitin and were identified by amino acid sequence analysis of the purified proteins. Actin and ubiquitin were found associated with all purified retroviruses examined, which included murine, feline, and nonhuman primate type C viruses; murine type B virus; primate type D virus; primate and ungulate lentiviruses; bovine leukemia virus; and human T cell lymphotropic virus type I (HTLV-I). However, analysis of HIV-1_{MN}, HIV-1_{IIIB}, HIV-2_{ISY}, SIV_{Mne}, and SIV_{Cat} (as in Fig. 1) revealed substantial amounts of β_2m and HLA DR, showing that these cellular proteins were common to many strains of primate immunodeficiency viruses. Purified proteins, including β_2m and HLA DR, were quantitated by amino acid analysis. For each virus, the molar ratio of β_2m and HLA DR to Gag protein was approximately 0.15 to 0.2. Assuming 2500 to 3000 Gag molecules per virion (*3*), we calculated that there are between 375 and 600 molecules of β_2m and HLA DR (α and β chains) per virion in the virus preparations. These calculations suggest that these specific cellular antigens outnumber the molecules of the envelope glycoprotein, gp120, on the virus [it has been previously estimated that there are approximately 216 molecules of gp120 per virion (*5*)]. We recovered more β_2m and HLA DR than gp120 during rp-HPLC purification of immunodeficiency viruses.

β_2m is a highly conserved protein that noncovalently associates with the HLA class I polymorphic proteins, termed heavy or α chain, to form the heavy (α) and light (β_2m) chain combination that is expressed on the surface of most, if not all, nucleated mammalian cells. Bovine β_2m from fetal bovine serum will exchange with β_2m complexed with the α chain on the surface of cells (*6*), and both human and bovine β_2m were found in virus preparations (Fig. 1). However, we have detected only trace amounts of α chain (copurifying with actin, Fig. 1B) in fractions from the rp-HPLC–purified viruses, suggesting that the amounts of β_2m in the virus preparation may be in excess of the amounts of HLA class I α chain.

HLA class II antigens are primarily expressed on cells of the monocyte-macrophage lineage, dendritic cells, B cells, and activated T cells and consist of heterodimers encoded by distinct gene regions (HLA DR, HLA DQ, or HLA DP) of the

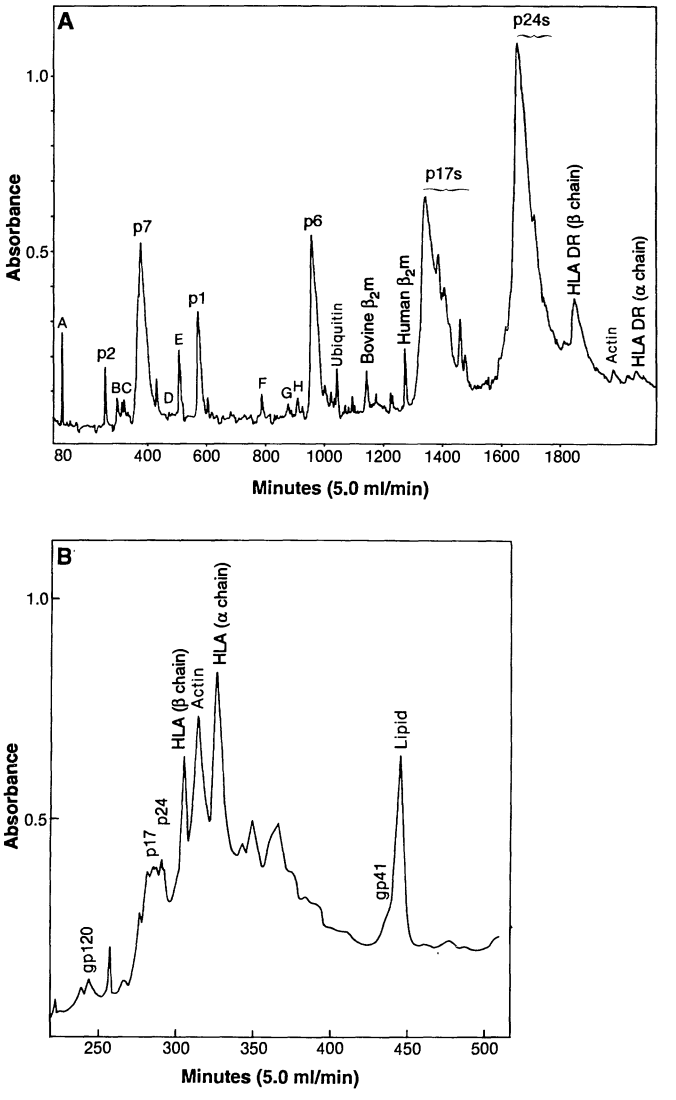

Fig. 1. Protein separation by rp-HPLC. Approximately 175 mg of purified HIV-1_{MN} was disrupted, and the viral proteins and peptides were separated by preparative rp-HPLC as described (*5*). Eluted proteins and peptides were detected by ultraviolet absorption at 206 nm. Proteins were eluted at room temperature with a gradient of increasing acetonitrile concentration (0 to 60%) (**A**). The temperature was raised to 50°C, and elution continued with a 0 to 100% 1-propanol gradient (**B**). HIV-1 proteins are designated as p2, p7, p1, p6, p17, and p24 (Gag proteins) and as gp120 and gp41 (envelope proteins) (*5*). Other peptides with Gag sequences are labeled as A through H. *Pol*-coded products were also found (in minor peaks) but are not labeled. The β_2 microglobulins from bovine and human sources are designated as Bovine β_2m and Human β_2m, respectively. Ubiquitin, HLA DR (β chain), actin, and HLA DR (α chain) are also indicated.

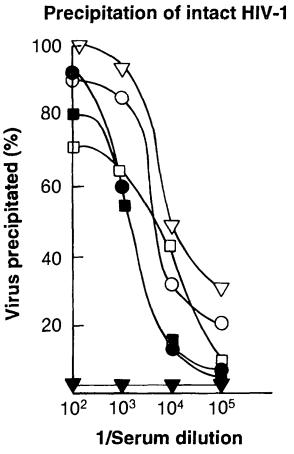

Fig. 2. Precipitation of virus by antisera to cellular proteins. Twenty microliters of each serum at the appropriate dilution was mixed with 280 μl of phosphate-buffered saline and 100 μl of HIV-1_{MN}. The mixture was incubated for 1 hour at 37°C and then overnight at 4°C; after the incubation, 50 μl of 10% *Staphyloccus aureus* was added to facilitate precipitation of immune complexes. After a 30-min incubation at room temperature, pellets were prepared by centrifugation for 30 min at 3000 rpm with a Beckman J-6M centrifuge. Approximately 50 μl of supernatant was removed, added to 950 μl of 2% Triton X-100, and incubated for 1 hour at 37°C. We measured HIV-1 p24 in this lysate by using a DuPont HIV-1 p24 antigen capture assay, according to manufacturer's instructions. The antisera used in the assay are listed in Table 1. These include antisera to H9 cells (▽), β_2m (○), HLA DR (●), HLA class I (■), HLA class I α chain (□), and negative sera (actin, CD4, mouse immunoglobulin G, and bovine serum albumin) (▼).

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major histocompatibility complex. Only HLA DR was present in virus preparations, which is in agreement with the report that HLA DR but not HLA DP or HLA DQ is found on virus by fluorescence-activated cell sorter analysis (*7*). Infection of CEM cells with HIV-1 is known to up-regulate expression of HLA DR (*8*). To see if up-regulation of HLA class II on H9 cells after HIV-1 infection accounted for the preferential incorporation of HLA DR into the virus, we determined the relative concentrations of HLA DR, HLA DQ, and HLA DP on HIV-1–infected and uninfected H9 cells by means of flow cytometry as described (9) (Table 1). Both the HIV-1–infected and uninfected H9 cells expressed HLA DR, HLA DQ, and HLA DP in the same relative concentrations, indicating that infection had not selectively altered the concentrations of the antigens on the cell surface. The presence of HLA DQ and HLA DP on the surface of HIV-1–infected H9 cells, but not in purified viruses, indicates a selective incorporation of HLA DR over HLA DP and HLA DQ.

Table 1. Analysis of relative concentrations of HLA antigens in HIV-1–infected and uninfected cells. Fluorescein-labeled monoclonal antibodies against determinants common to products of alleles at class I loci (HLA A, B, and C) and class II loci (HLA DR, DQ, and DP) were added at saturating concentrations to the infected and uninfected cells. The mean fluorescence was determined by flow cytometry. Isotypically matched monoclonal antibodies served as controls.

Cells	Mean fluorescence				
	HLA class II			HLA class I	Control
	DR	DP	DQ	ABC	
HIV-1–infected H9	165	94	50	68	5
Uninfected H9	164	76	43	72	5

Table 2. Neutralization of SIV and HIV-1. Neutralization assays were performed on the AA-2 CL 1 cell line as described (*1*). HIV-1_{IIIB} and SIV_{Mne} were grown in Hut 78 cells. Antiserum to H9 cells was prepared by immunization of a rabbit with "mock virus" prepared from uninfected H9 cells (*4*); anti–human β_2m was obtained from Polysciences; anti–HLA class I was monoclonal antibody (W6/32) from DAKO; and anti–HLA DR (R.DRAB1) was provided by P. Cresswell (*19*). Neutralization titer is expressed as the serum dilution that resulted in 50% or greater reduction in giant cell formation on the AA-2 CL 1 cells infected with SIV or HIV-1. NT, not tested.

Serum	Neutralization titer	
	SIV_{Mne}	HIV-1_{IIIB}
Anti-H9	320	80
Anti-β_2m	704	1408
Anti–HLA DR	640	1280
Anti–HLA class I	1280	1280
SIV_{Mne}-infected macaque	5632	<22
HIV-1–infected human	NT	1408
Normal rabbit	22	<22
Normal macaque	<22	NT
Normal human	22	22

To determine if these viral-associated cellular antigens were physically attached to HIV-1, we tested antisera specific for β_2m and HLA DR for their ability to precipitate intact virus. Antisera to uninfected H9 cells, β_2m, HLA DR, HLA class I, and HLA class I α chain efficiently precipitated intact virus (Fig. 2), indicating that cellular proteins were physically attached to the virus surface. Precipitation of intact virus with monoclonal antibodies to class I α chain (HC-10) and to conformationally dependent epitopes on HLA class I (W6/32) indicates that some of the viral-associated β_2m is complexed with α chain, forming complete HLA class I molecules on the surface of most of the virus particles. Antisera to actin, CD4, mouse immunoglobulin G, and bovine serum albumin did not precipitate the virus. Furthermore, monoclonal antibodies to HLA DR (but not to HLA DP or HLA DQ) precipitated intact HIV-1 (*10*).

Neutralization of HIV-1 and SIV infection by specific antisera has proven useful for identification of viral and cellular proteins involved in infection and for localization of regions of the protein that interact during the infection process. To determine if the viral-associated β_2m and HLA DR proteins were involved in virus infection, we tested antisera to β_2m and HLA DR in vitro for their ability to neutralize HIV-1 and SIV infection. Antisera to β_2m and HLA DR efficiently inhibited HIV-1 and SIV infection, as shown in Table 2. Monoclonal antibody to β_2m, B2g2-2 (*11*), also neutralized infection of human peripheral blood lymphocytes by HIV-1_{NDK} and HIV-1_{LAV} propagated in CEM cells (*12*). Because β_2m and HLA DR are expressed on the surface of cells, it was necessary to determine whether the neutralization was due to the binding of antisera to antigens on the cells or on the virus. We preincubated cells with antisera to β_2m or with a monoclonal antibody (OKT4a) that inhibits gp120-CD4 interactions by binding to a reactive site on the CD4 (*13*); the cells were then washed to remove antisera, and HIV-1 was added to the cells. Whereas no inhibition of HIV-1 infection was seen with anti-β_2m, and only high concentrations of anti-DR sera inhibited HIV-1 infection, preincubation with OKT4a completely prevented virus infection (*14*), suggesting that the β_2m and HLA DR on the virus, and not on the cell, were the primary targets for neutralization by the respective antisera. Neutralization of HIV-1 and SIV infection by antibodies to β_2m and HLA DR is further evidence that these cellular proteins are physically associated with HIV-1 and SIV and suggests that these viral-associated cellular proteins are involved in the infection process.

We previously identified HLA DR molecules in sucrose density gradient–purified HIV-1 (*15*). Also, it has been shown that antisera to selected cellular antigens react with HIV-1 and SIV (*16*). However, we now implicate specific viral-associated cellular proteins in the infection process. The importance of understanding the infection process of immunodeficiency viruses is emphasized by differences in the pathogenic potential of HIV-1 isolates that appear to be attributable to efficiency of virus entry (*17*). We propose that β_2m, HLA DR, HLA class I, and gp120 on the immunodeficiency viruses interact with specific ligands on the cell (similar to binary interactions between T cells and antigen-presenting cells), allowing the formation of an "adhesion patch" of clustering molecules on the cell surface. This adhesion patch may then serve to fuse the virus and cell membranes and allow the viral core–associated genome to penetrate the cell. The presence of HLA DR on immunodeficiency viruses also has significant implications for our understanding of the pathogenesis of HIV-1 infection. The function of HLA class II molecules is to present antigens, in the form of peptides, to T cell receptors (TCR) in the initiation of cellular and humoral immune responses of the host. Inappropriate signaling after class II binding to TCR can cause apoptosis (or programmed cell death) of the TCR-bearing cell, which has been proposed as the pathological mechanism for induction of immunosuppression in AIDS (*18*). We propose that binding of viral-associated HLA DR to the TCR on CD4 cells may eliminate the CD4 cells by apoptotic mechanisms.

Our evidence suggests that consideration of immunodeficiency viruses should now be expanded to include cellular proteins as an integral and functioning part of the viral envelope. Whether the cellular proteins are components of the viral envelope acquired during budding or are acquired after budding by specific associations is an important mechanistic consideration that can now be addressed. In any case, it is likely that immune responses to HLA DR, HLA class I, or β_2m (or to all three cellular proteins) were involved in protection against SIV infection after immunization with uninfected human cells (*2*). We propose that the cellular proteins associated with immunodeficiency viruses play a cen-

tral role in infection and pathogenesis. These proteins should be considered in elucidation of the steps involved in infection, design of vaccines, preparation of experimental virus-challenge stocks, and determination of the pathological mechanisms of immunodeficiency viruses.

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Protective Effects of a Live Attenuated SIV Vaccine with a Deletion in the *nef* Gene

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Vaccine protection against the human immunodeficiency virus (HIV) and the related simian immunodeficiency virus (SIV) in animal models is proving to be a difficult task. The difficulty is due in large part to the persistent, unrelenting nature of HIV and SIV infection once infection is initiated. SIV with a constructed deletion in the auxiliary gene *nef* replicates poorly in rhesus monkeys and appears to be nonpathogenic in this normally susceptible host. Rhesus monkeys vaccinated with live SIV deleted in *nef* were completely protected against challenge by intravenous inoculation of live, pathogenic SIV. Deletion of *nef* or of multiple genetic elements from HIV may provide the means for creating a safe, effective, live attenuated vaccine to protect against acquired immunodeficiency syndrome (AIDS).

There are good reasons for believing that development of an effective vaccine for AIDS will be a difficult task. Infection of humans with human immunodeficiency virus type–1 (HIV-1) and of rhesus monkeys with SIVmac is fatal most or all of the time despite an apparently strong host immune response to the infecting virus. Infected individuals maintain vigorous humoral and cellular immune responses for months or years only to succumb eventually to the virus. The ineffective nature of the natural immune response suggests that a vaccine will have to provide highly stringent protective immunity, perhaps even sterilizing immunity, in order to achieve protection. These difficulties are compounded by the large number of HIV-1 strains that are non– or minimally cross-neutralizing.

The predicted difficulty in achieving protection against HIV and SIV has been borne out to varying degrees by vaccine trials in animal models. Inactivated whole virus has protected rhesus monkeys against challenge by live, pathogenic SIV (*1–4*) but primarily under highly specific conditions that use human cells for the production of both vaccine antigen and challenge virus. Even formalin-fixed, uninfected human cells can provide protection under these conditions (*5*). Apparently, an immune response to some cellular antigen or antigens is critical for this protection. The inactivated whole virus vaccine approach has been largely unsuccessful against SIV grown in rhesus monkey lymphocytes (*6, 7*). Priming with vaccinia recombinants followed by boosting has protected rhesus monkeys against challenge by homologous cloned SIV (*8*), but little or no protection has been observed against homologous uncloned SIV (*9–11*). Tests of several products have shown only limited success in chimpanzee trials (*12*). What is most disappointing about these studies is that the numerous failures have occurred despite extensive efforts to maximize, in an unrealistic fashion, the likelihood of vaccine protection. The vast majority of studies have used a minimal dose of challenge virus, matched to the strain used for vaccination, at or near the peak of vaccine-induced immune response.

We investigated an approach that uses live attenuated SIV as a vaccine. Six rhesus monkeys that were infected with cloned SIVmac239 that contained a constructed deletion in the auxiliary gene *nef* have maintained extremely low virus burdens and normal $CD4^+$ lymphocyte concentrations and have remained healthy for more than 3 years after experimental inoculation with the mutated virus (*13, 14*). Eleven of twelve rhesus monkeys infected with wild-type SIV in parallel have died over this same period. The rhesus monkeys infected with SIVmac239/*nef*-deletion have shown no clinical signs whatsoever over the entire period of observation.

Four of the rhesus monkeys infected with *nef*-deleted SIV were challenged with

Table 1. Challenge of rhesus monkeys immunized with SIV deleted in *nef*. All four rhesus monkeys received a single inoculation of SIVmac239/*nef*-deletion 2.25 years before challenge. Attempts to recover SIV from 10^6 PBMCs on the day of challenge yielded negative results (dashes). The titers of neutralizing antibodies were measured on the day of challenge (*1*).

Rhesus monkey	SIV recovery	Antibody titer	Challenge virus
353-88	–	1:1280	SIVmac239/*nef*-open
397-88	–	1:1280	SIVmac239/*nef*-open
71-88	–	1:320	SIVmac251
255-88	–	1:2560	SIVmac251

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