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Comparison of nucleic acid and protein immunization for induction of antibodies specific for HIV-1 gp120

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SUMMARY

We have compared the antibody response to HIV-1 gp120 type LAI in mice immunized with either a gp120 expression plasmid or with baculovirus-derived recombinant gp120 (rgp120) formulated with Freund's complete adjuvant, TiterMax, Alum, Ribi R-700, AF-A or QuilA. DNA immunization resulted in variable levels of antibody, with endpoint titres ranging from 10^4 to 10^5, whereas mice immunized with rgp120 mixed with Ribi R-700, AF-A or QuilA produced antibody levels with endpoint titres > 10^5. Both types of immunization failed to elicit antibodies able to recognize denatured rgp120. The V3 region was immunogenic in animals immunized with nucleic acid, whereas only a few animals immunized with recombinant protein produced antibodies specific for V3 or other linear epitopes, irrespective of the adjuvant used. These data suggest that the immunogenicity of gp120 is dependent upon the mode of antigen delivery, and that *in vivo* expressed gp120 following nucleic acid immunization elicits, at least with respect to V3, an antibody response which more closely reflects that seen following natural infection in man.

Keywords DNA vaccination vaccine HIV gp120 IIIB IgG subclasses AIDS

INTRODUCTION

Induction of protective immunity to pathogens depends to a great extent on the ability of a vaccine to initiate an immune response that can either neutralize the pathogen before invasion of the host cells or eliminate the infected cells. The most appropriate type of protective immune response depends on the life cycle of the pathogen, and whether the vaccine is intended for preventive or prophylactic use. Recombinant subunit vaccines invariably require the additional immunostimulatory potential of an adjuvant, which may then influence the type of immune response induced. However, many of the adjuvants currently available carry unacceptable side-effects which severely limit their use in man. Alternative means of vaccine delivery have therefore been sought. Recently a new approach based upon direct injection with nucleic acid encoding the gene of interest has been developed [1,2], a procedure which induces both cell-mediated and humoral immune responses [3].

The outer envelope glycoprotein (gp120) of HIV-1 is the principal target for the neutralizing antibody response, and has therefore been considered a major candidate for a recombinant subunit vaccine. We have compared the specific antibody response to HIV-1 gp120 (clone BH10/LAI) induced in mice immunized with recombinant gp120 protein to that induced following immunization with plasmid DNA encoding the gp120 open reading frame (ORF).

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MATERIALS AND METHODS

gp120 antigens and monoclonal antibodies

The MoAbs used in this study are listed below, with their epitopes shown in brackets after the clone nomenclature: 11/65a (C1-aa102–121); 11/41a (C5-aa471–491); 11/4c (V2-aa152–181) [4,5]; 11/68b (V1/V2 discontinuous) [4,5]; 10/36c (V3-aa311–321); 10/54ow (V3-aa311–321); 11/85b (V3-aa311–321); ICR41 (V3-discontinuous); ICR38.1a (C4-aa427–436) [6]; ICR39.13 g (CD4 binding site discontinuous) [7] and 8/19b (gp120 discontinuous) [8].

Baculovirus-derived recombinant HIV-1 gp120 LAI (rgp120, clone BH10) and peptides representing gp120 sequences were used to monitor specific IgG responses. Peptides to the following gp120

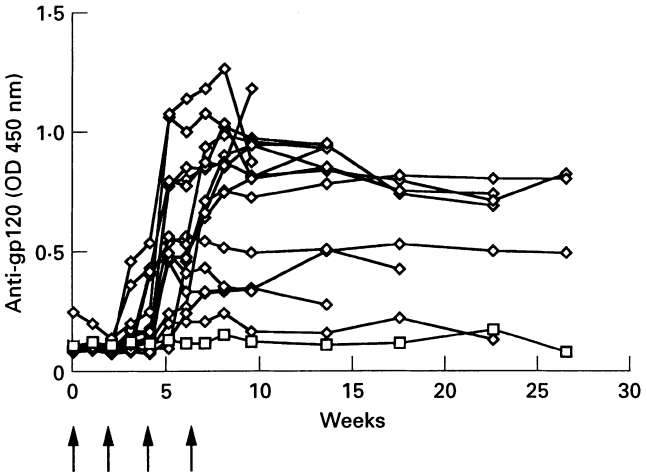

Fig. 1. The gp120-specific immune response in DNA-immunized mice. Sera (diluted 1:100) from d×b mice injected with pEE14.tPAgp120.3 on days 0, 14, 28 and 42 (arrows) were assayed in a capture ELISA for reactivity with rgp120. Each line represents an individual mouse. Sera from d×b mice injected with control plasmid pEE14 (□) did not react with rgp120.

regions were used: 740.8 (71EQMHEDIISLWDQSLKPCVK) specific for the first conserved region (C1); 729.3 (301CTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHCN) specific for the third variable region (V3); 709 (409NMWQKVGKAMYAPPISG) specific for the C4 region; and 710 (489VKIEPLGVAPTKAKRRVVQREKR) specific for the C-terminal (C5) region of gp120.

The peptides were obtained from the MRC-AIDS Directed Programme (ADP).

Protein immunization

Six-week-old male CBA/Ca×BALB/c (k×d haplotype) or BALB/c×C57Bl/6 (d×b haplotype) mice were immunized three times (days 0, 30 and 90) with 1 μg rgp120 together with adjuvant in a 100-μl volume injected subcutaneously in two 50-μl depots. Sera were collected pre-immunization and on days 28, 45 and 100 post-immunization.

Adjuvants

The following adjuvants were used: Freund's complete adjuvant (FCA) followed by Freund's incomplete adjuvant (FIA; Sigma, Poole, UK) [9]; Hunter's TiterMax (Sigma) [10]; AF-A (IDEC Pharmaceuticals, La Jolla, CA) [11]; Ribi R-700 adjuvant system (Ribi R-700 ImmunoChem Research, Hamilton, MA) [12]; QuilA (Superfos, VedbÆk, Denmark) [13], and alum [14]. Each adjuvant was formulated with antigen according to the supplier's instructions, and in the case of alum the gp120 was dialysed against 0·9% NaCl to remove phosphate ions and precipitated with aluminium potassium sulfate ($AlK(SO_4)_2.12H_2O$).

Nucleic acid immunization

pEE14 [15], an expression vector derived from pEE6 [16], contains the mammalian selection marker glutamine synthetase and expresses foreign genes under the control of the human CMV promoter. The plasmid pEE14.tPA.gp120.3 contains the coding region of HIV-1 gp120 LAI linked to the signal sequence of human tissue plasminogen activator. Both plasmids were kindly provided by Dr Paul Stephens (Celltech, Slough, UK).

Six-week-old female CBA/Ca (k haplotype) ($n=8$), CBA/Ca×BALB/c (k×d haplotype) ($n=8$) or BALB/c×C57Bl/6 (d×b haplotype) ($n=8$) mice were injected with 100 μg bupivacaine (Astra Pharmaceuticals Ltd, Kings Langley, UK) intramuscularly 24 h before the injection of DNA [17]. Qiagen column (Qiagen Ltd, Dorking, UK) purified, pEE14.tPAgp120.3 or control plasmid pEE14 were dissolved in 0·9% NaCl at 1 mg/ml, and 100 μl (100 μg) DNA were injected at weeks 0, 2, 4 and 6 into the right soleus muscle of the mice. Blood (50 μl) was taken into heparin the day before each DNA injection, and mice were bled out 15 days after the final injection.

Quantification of gp120-specific antibody response

Nunc MaxisorpC microtitre plates were incubated at 4°C overnight with sheep anti-gp120 capture antibody, raised against a peptide representing the C-terminus of gp120 (D7324; Aalto Bioreagents, Dublin, Republic of Ireland), at 3 μg/ml with 100 μl/well in 0·05 M carbonate-bicarbonate buffer pH 9·6. The plates were washed once in PBS containing 0·05% Tween 20 (PBS–T), followed by blocking with 200 μl of 10% fetal calf serum (FCS) in PBS for 2 h at room temperature. Subsequent washes were three times with PBS–T. rgp120 (1 μg/ml) in PBS–T/1% bovine serum albumin (BSA) (assay buffer) was allowed to bind to the D7324-coated plates for 4 h at 37°C. After washing, 100 μl mouse serum, diluted in assay buffer, were added to each well and incubated for 16 h at 4°C. Following further washes, gp120-bound antibodies were detected with goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma) and then the substrate *p*-nitrophenylphosphate (Sigma) in 0·05 M carbonate-bicarbonate buffer pH 9·6, 2 mM $MgCl_2$. When the reaction of monoclonals used as positive controls reached approximately A410 = 0·8, the plate was read using a Dynatech MR5000 ELISA plate reader. End-point titres were determined as the serum dilution which gave a signal equal to the mean + 2 s.d. of a group of nine sera from unimmunized control mice. Responses were analysed using Minitab software to carry out a one-way analysis of variance.

Denaturation of rgp120

The rgp120 was denatured essentially as described by Moore & Ho [18]. rgp120 (10 μg) was boiled for 5 min in PBS containing 10% FCS, 1% SDS, 50 mM DTT and then diluted into nine volumes of PBS containing 10% FCS and 1% Nonidet P40 to bring the rgp120 concentration to 1 μg/ml. Denatured antigen was used in the ELISA assay described above.

Anti-peptide response

The antibody levels to the individual linear peptides were determined by ELISA. Peptides were coated directly on to plates at 3 μg/ml in 0·05 M carbonate-bicarbonate buffer pH 9·6, at 4°C for 16 h. The ELISA was then carried out as described above. MoAbs recognizing specific peptides served as positive controls to confirm the presence of the peptide, and their binding was detected using an antibody to rat light chains conjugated to alkaline phosphatase (Sigma).

RESULTS

DNA immunization

As illustrated in Fig. 1 for the d×b haplotype, there was a marked

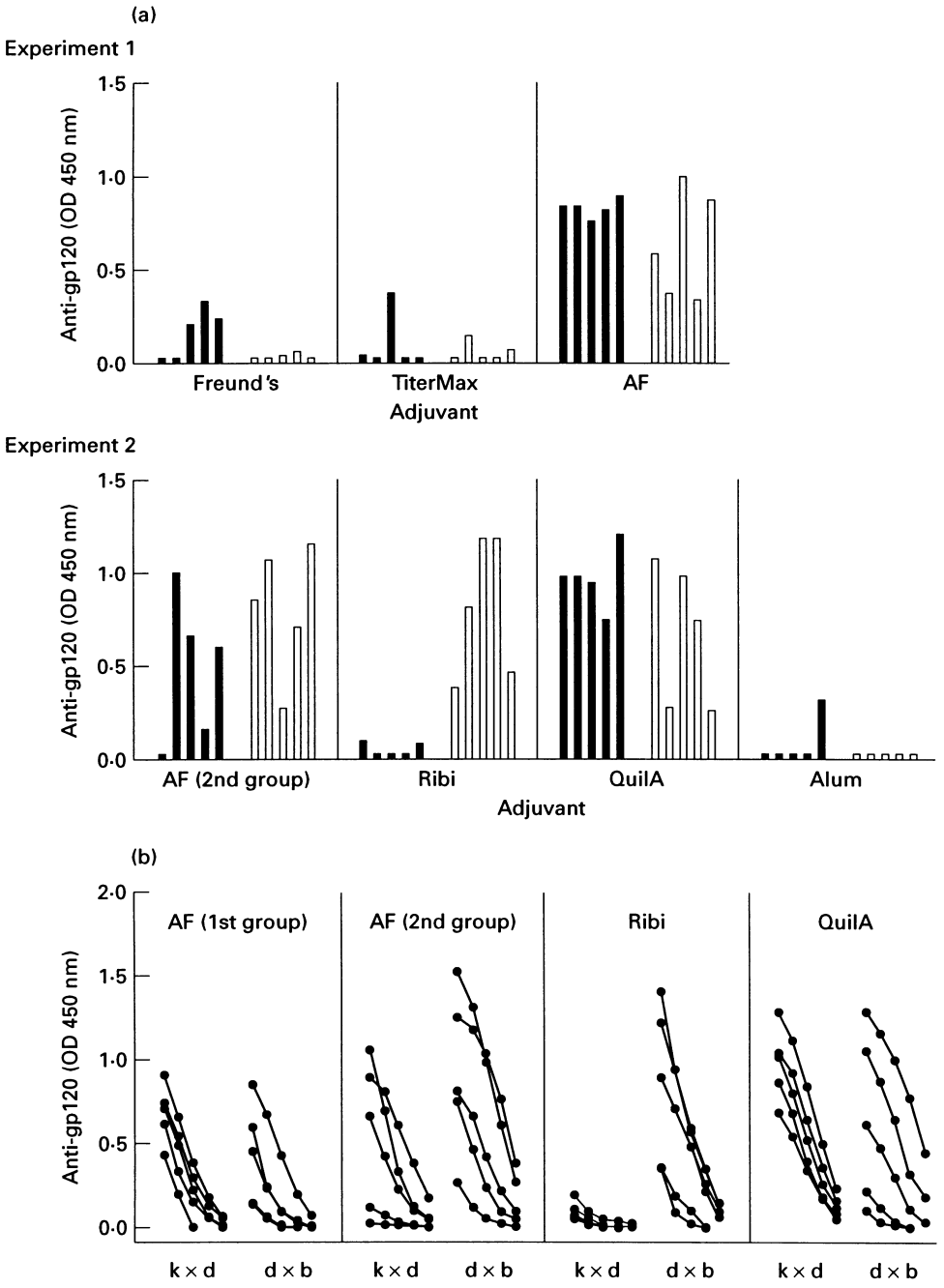

Fig. 2. The gp120-specific immune response in rgp120-immunized mice. (a) Sera (diluted 1:1000) from k × d haplotype ($n = 5$) (■) or d × b haplotype ($n = 5$) (□) mice immunized with baculovirus-produced rgp120 IIIB (clone BH10) formulated with different adjuvants were assayed in a capture ELISA for reactivity with rgp120. Each line represents serum taken at 15 days after the last boost from an individual mouse. Results from two separate experiments are shown. (b) Titration curves for sera which showed positive reactivity with gp120. Sera were diluted 1:1000, 1:3000, 1:9000, 1:27 000 and 1:81 000 from left to right in each set of curves.

variation in the time of onset of a detectable antibody response following injection with the gp120 expression vector pEE14.t-PAgp120.3. The gp120-specific IgG also varied between individual mice, with endpoint titres ranging from 10^3 (a few animals) to 10^5, with a few animals producing negligible levels of gp120-specific antibodies. Comparable variation in antibody responses was observed in mice of various haplotypes immunized with the same expression plasmid (data not shown).

Protein immunization

There was a marked difference in the IgG levels induced with the different adjuvants (Fig. 2), consistent with the results reported by others [19]. The six adjuvants used in this study fall into two groups. Alum, TiterMax and FCA induced very low antibody levels. In contrast, AF-A, Ribi R-700 or QuilA produced a response with endpoint titres generally in the 10^5–10^6 range. Immunogen formulated with Ribi R-700 showed a marked haplotype

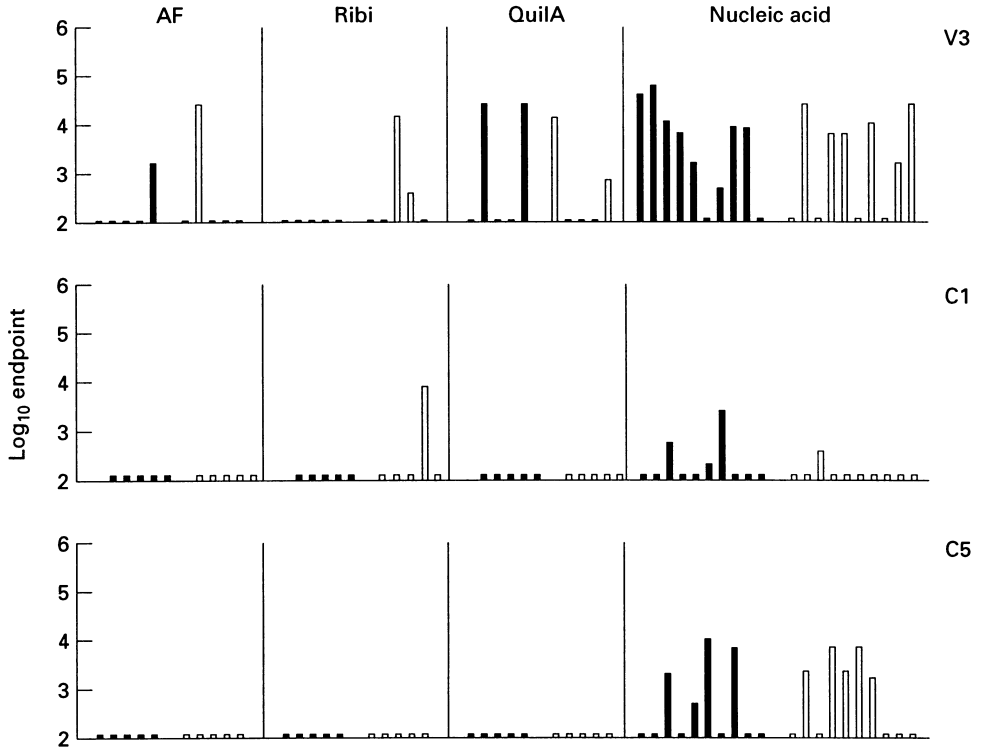

Fig. 3. Comparative peptide reactivity of sera from DNA- and protein-immunized mice. Sera from k×d (■) and d×b (□) mice immunized with rgp120 in adjuvant or with pEE14.tPAgp120.3 plasmid were titrated for their reactivity with V3, C1 and C5 peptides in a capture ELISA. Rat MoAbs were used as controls on each plate. The sequence of the peptides is given in Materials and Methods.

dependency with reduced antibody titres in the k×d haplotype compared with d×b haplotype ($F = 14{\cdot}84$, $P = 0{\cdot}005$). For all the other adjuvants there was no statistically significant difference in specific IgG titres between the two F_1 haplotypes. The endpoint titres of gp120-specific antibodies obtained with AF-A, Ribi R-700 and QuilA were generally greater (10^5–10^6) than obtained following DNA immunization (around 10^4) ($F = 12{\cdot}67$, $P = 0{\cdot}001$).

Comparison of the immune response following DNA and protein immunization

Since the use of adjuvants may lead to partial denaturation of the immunogen, we compared the epitope specificity of antibodies produced following nucleic acid and protein immunization. Moore & Ho [18] previously reported that antibodies produced following natural HIV infection predominantly recognize discontinuous epitopes on gp120. Sera from immunized mice demonstrated no or minimal recognition of denatured rgp120 (data not shown). However, since MoAb 11/41a recognizing a linear epitope in the C5 region was also unable to recognize the denatured gp120, it is likely that the reduced polypeptide chain in the detergent environment folds into a different molten state, which does not expose all the linear epitopes present on native gp120.

To characterize further the immunogenic epitopes, the immune sera were assessed for their ability to bind a series of peptides covering different regions of gp120. One of the immunogenic neutralizing determinants of gp120 is located in the third variable region, V3 [20]. To assess the immune response to linear epitopes within V3, serum reactivity to a 36-mer peptide spanning the entire V3 region was followed. Very few sera from mice immunized with rgp120, independent of the adjuvant used, contained detectable levels of V3-specific antibodies (Fig. 3). In contrast, most sera from DNA-immunized mice contained antibodies reactive with the V3 peptide, with endpoint titres in the 2×10^3–4×10^4 range. These levels are comparable to the endpoint titres obtained for the native rgp120 molecule. Although there is no difference in V3 peptide IgG endpoint titre between protein and nucleic acid immunization ($F = 0{\cdot}02$, $P = 0{\cdot}89$), bearing in mind that the endpoint IgG reactivity with the whole gp120 molecule is 10 times higher in protein-immunized sera than in nucleic acid sera, the relative peptide reactivity of nucleic acid serum is increased over that of protein. These data suggest that DNA immunization has the potential to induce strong neutralizing antibody responses to the virus. However, the sera from the DNA-immunized animals failed to neutralize the molecular cloned virus BH10 (data not shown).

Some of the sera from the nucleic acid-immunized mice also contained antibodies specific for linear sequences in the C1 and C5 regions of gp120. This is in contrast to the results obtained with the protein-immunized animals, where only one animal produced antibodies reactive with the C1 peptide and none with the C5 peptide (Fig. 3). Finally, sera were tested for reactivity with a truncated gp120 molecule containing the N-terminal and V1/V2 regions of the envelope protein [5]. It is interesting to note that the V1/V2 region has been reported to be a target for neutralizing antibodies and is immunogenic in infected individuals [4]. A very restricted number of sera from the protein-immunized animals contained V1/V2 reactivity, while none of the sera from the DNA immunized mice contained detectable antibody levels (data not shown).

Antibody subclasses

The gp120-specific serum response from the DNA- and protein-immunized mice were typed with IgG1- or IgG2a-specific

enzyme-conjugated antibodies. Most of the sera, irrespective of the mode of immunization, contained predominantly IgG1 antibodies, with the exception of the occasional mouse immunized with protein mixed with either AF-A or QuilA, where higher levels of IgG2a gp120-specific antibodies were seen (data not shown). These data suggest that, irrespective of the type of immunization, a primarily Th2-mediated humoral immune response was induced.

DISCUSSION

We have compared the antibody response following injection of mice with an HIV-1 gp120 expression plasmid to that produced following immunization with purified recombinant baculovirus-produced gp120. Immunization with DNA induced a long lasting antibody response [21], with endpoint IgG titres of 1:8000–1:50 000 irrespective of the mouse strain used. Approximately 90% of animals produced detectable levels of specific antibodies, a response rate comparable to that reported by others [19,22–25]. The heterogeneity in IgG levels seen in the DNA-immunized mice appears to be independent of bupivacaine pretreatment, since comparable variation was seen when cardiotoxin was used as a facilitator [21]. When using its natural viral signal sequence, HIV-1 gp120 in cell lines is synthesized and secreted at a very low level, although the *env* gene with a signal sequence from herpes simplex virus glycoprotein, honeybee mellitin, IL-3 or human tissue plasminogen activator promotes high expression of gp120 in eukaryotic systems ([26–29], P. Stephens, personal communication). However, mice injected with an HIV-1 gp120 construct possessing the HIV viral signal sequence induced an equally heterogeneous immune response to that obtained in animals immunized with a gp120 gene containing the signal sequence of tissue plasminogen activator [21]. It is possible that the observed variation is due to the uncontrolled way in which the DNA is taken up. However, since the recombinant antigen synthesized *in vivo* lacks adjuvant, it may be presented suboptimally to the immune system.

The antibody titres in the DNA-immunized mice should be contrasted with the titres obtained following tertiary immunization with rgp120 in adjuvant. The specific antibody levels induced showed a strong dependency on the adjuvant chosen. Consistent with our results, gp120 formulated with alum has been shown to induce a weak antibody response in chimpanzees [30], rhesus macaques [31], guinea pigs [32] and mice [19,33]. Several authors have suggested that gp120 interaction with alum is weak and that a number of commonly used buffers can further weaken this interaction [34]. However, the rgp120 preparation used in our study was dialysed extensively against 0·9% NaCl to remove any traces of phosphate ions before precipitation with aluminium sulfate. In addition, other authors [19,35] have reported that rgp120 formulated with FCA fails to induce a strong antibody response, again consistent with our results. Genetic differences in gp120-specific antibody responses have been reported previously [19], therefore we used both $F_1(d \times b)$ and $F_1(k \times d)$ mice to ensure broad MHC haplotype specificities. rgp120 formulated with Ribi R-700 failed to elicit significant antibody levels in $k \times d$ mice. In contrast, the genotype had no influence on the antibody response in DNA-immunized mice.

Irrespective of the immunization regime, immunoreactive sera contained antibodies capable of recognizing discontinuous epitopes present on native rgp120. One might anticipate that denatured protein would react primarily with antibodies recognizing linear determinants, but some MoAbs specific for linear epitopes failed to recognize the denatured protein, suggesting that the denaturation procedure allowed the polypeptide chain to assume a different conformation. However, when sera were tested for reactivity with selected gp120 peptides, very few sera from the protein-immunized animals displayed detectable reactivity, irrespective of the adjuvant used. In contrast, more of the antisera from the DNA-immunized mice contained antibodies specific for C1, V3 and C5 peptides. Differences in the glycosylation pattern of a given antigen may affect the protein conformation [36,37] as well as antigen presentation [38–40]. The baculovirus-produced gp120 will almost certainly exhibit a different glycosylation pattern to that produced endogenously *in vivo*. It is known that the ability to stimulate cytokine release from macrophages depends on the source of recombinant gp120 [36] and that a slightly different CD4 T cell response can be obtained in mice immunized with galactosidase-modified rgp120 derived from either chinese hamster ovary (CHO) cells or from baculovirus-infected insect cells [38]. The variation in levels of antibodies to the V3 and C1 peptides between mice immunized with baculovirus-produced rgp120 and those immunized with DNA could therefore be a result of structural differences in the gp120 presented to the immune system.

In this context, it is interesting to note that human sera from naturally infected individuals also react with native rather than denatured gp120 [18], with a significant fraction of the antigen-reactive antibodies recognizing V3 [18]. This is in marked contrast to the analysis of antibodies elicited in humans immunized with baculovirus rgp120 or gp160 formulated with alum. In this case, sera react preferentially with carboxy-methylated gp120 rather than with native antigen, indicating that the rgp120/gp160-alum immunogen preparation primarily elicits antibodies directed against linear epitopes [41]. However, it is possible that the carboxy-methylated gp120 protein was able to fold into a conformation relatively similar to the native protein, whilst the reduced and denatured protein may refold into a number of different molten states in the presence of the detergent.

The V3 region constitutes an immunogenic linear epitope capable of producing neutralizing antibodies in naturally infected individuals [42]. However, we failed to observe V3-reactive antibodies in the majority (22 out of 30) of sera from protein-immunized mice irrespective of haplotype. In agreement with our results, Bristow *et al.* [35] reported poor immunogenicity of the V3 region in gp120-FCA-immunized mice. Furthermore, this was consistent with results obtained by Bomford *et al.* [19], where only two of 10 sera from immunized BALB/c mice contained V3-specific antibody levels above background, whereas six out of 10 CBA mice immunized with gp120 formulated with QuilA or MDP/Pluronic emulsion developed a V3-specific antibody response with endpoint titres of 10% or less of the total gp120 titres. In contrast, most sera from DNA-immunized animals in the present study contained V3 antibodies. It is therefore tempting to speculate that intramuscular injection of expression plasmids containing the gene for a glycosylated protein such as gp120 will produce recombinant proteins that are glycosylated and folded in a native conformation. This may explain why DNA immunization appears to induce, at least in our hands, an immune response which, as in the natural infection and unlike protein immunization, is strongly directed towards V3.

In conclusion, mice immunized with a gp120 expression plasmid showed higher levels of V3-reactive antibodies than

mice immunized with various rgp120 protein/adjuvant formulations. *In vivo* expression of gp120 appears to present the antigen in a native form comparable to that seen during natural infection of humans. It can therefore be regarded as an alternative vaccination procedure, where problems of reversion and stability associated with attenuated vaccines do not apply.

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