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A recombinant human adenovirus expressing the simian immunodeficiency virus Gag antigen can induce long-lived immune responses in mice

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Human adenovirus type 5 can be used as a vector to elicit immune responses to antigens expressed from heterologous DNA sequences incorporated into the viral genome, for example in mice immunized intraperitoneally. We have used a recombinant adenovirus which expresses the p55^{gag} antigen of simian immunodeficiency virus to evaluate the nature and longevity of the response elicited when administered to mice by alternative routes which translate more readily to larger animals and man. In C57Bl/6 mice immunized orally with a single dose of virus, a majority of the animals which showed evidence of responding to the immunogen by producing an anti-adenovirus response also produced a plasma antibody response to Gag which persisted for more than 1 year and a Gag-specific cytotoxic T cell response that could be detected for at least 6 months. In a minority of similarly immunized responding animals, only a cytotoxic response to Gag was observed although both humoral and cellular responses to adenovirus antigens were seen; intranasal immunization produced a Gag-specific response similar to this latter pattern. These findings suggest that delivery of adenovirus recombinants orally or intranasally may be a useful strategy for eliciting long-term cytotoxic T cell memory responses in splenocytes to candidate vaccine antigens.

Introduction

Simian immunodeficiency virus (SIV) infection of macaques has proven to be an important animal model for vaccine development against human immunodeficiency virus (HIV). Protection against SIV has been demonstrated using a variety of inactive viral preparations (Dormont *et al.*, 1995). However, in these studies, protection was due to responses to xeno-host antigens in the vaccine and challenge virus preparations; protection was not observed against challenge virus grown in simian cells. Attenuated SIV vaccines have also demonstrated protection against virulent SIV challenge (Daniel *et al.*, 1992). However, the nature of the interaction of immunodeficiency viruses with their hosts makes the safety of this type of vaccine difficult to assess. Hence it is widely believed that a useful and safe vaccine will comprise some type of subunit preparation delivered either as inert antigen or in the form of a live recombinant vector.

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In designing candidate subunit vaccines there are still a number of obstacles to overcome. These include the identification of immunological epitopes on the virus, responses to which can confer protection, and the nature of the specific T and B cell responses generated which give rise to this protection (Vaslin *et al.*, 1994). Hu *et al.* (1992) have shown that inducing neutralizing antibody to recombinant envelope proteins can confer protection to homologous virus challenge. Extension of these studies (Hu, 1996) has shown the value of live vector delivery of the antigen, perhaps implying a role for cell-mediated immunity in protection. Another variable is the route of administration, which may alter the nature as well as the extent of the response to a given antigen. Using an inert preparation displaying Gag epitopes in macaques, Brookes *et al.* (1995) showed that the spectrum of T cell epitopes recognized was altered by changing the site of immunization. Similar material displaying Gag and Env epitopes could elicit protective responses via one route of delivery but not others (Lehner *et al.*, 1996).

Studies of SIV-infected macaques have indicated that the stimulation of cytotoxic T lymphocytes (CTL) is an important factor in maintaining freedom from disease (Bourgault *et al.*,

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1993); virus-specific CTL have also been implicated in protection from SIV infection (Gallimore *et al.*, 1995). Such responses are best elicited by live vaccines and one useful vector for the delivery of antigens in this form and the stimulation of an immune response is human adenovirus (Ad) (Imler, 1995). These viruses contain stable dsDNA that can be easily manipulated and inserts of up to 4 kbp can be inserted in the place of early region 3 (E3) without impairing viability (Bett *et al.*, 1993). Ad grows to high titres *in vitro* and vaccine strains have been shown to result in no adverse effects when administered orally to man (Top *et al.*, 1971). Ad serotypes replicate variously in the upper respiratory and gastrointestinal tracts where they have the ability to induce both humoral and cellular immune responses, making them useful carriers for vaccination (reviewed in Imler, 1995). Although few animals are permissive for the replication of human Ad vectors, immune responses can nevertheless be stimulated with these vectors in many different species of animal. In mice, protection has been demonstrated against subsequent challenge with a variety of viruses by prior immunization with an appropriate Ad recombinant, for example rabies (Prevec *et al.*, 1990), tick-borne encephalitis (Jacobs *et al.*, 1992), rotavirus diarrhoea (Both *et al.*, 1993), herpes simplex (Gallichan *et al.*, 1993), murine hepatitis (Wesseling *et al.*, 1993) and measles (Fooks *et al.*, 1995) viruses.

Previous work has shown that Ad expressing the Env or Gag antigens of human immunodeficiency virus (HIV) can induce immune responses in rhesus macaques (Prevec *et al.*, 1991) and chimpanzees (Natuk *et al.*, 1993). The variability of the *env* gene makes the Env protein a difficult antigen for a vaccine to target; however, the more conserved Gag protein is also a potent stimulator of both the cellular and humoral components of the immune system and may contain important protective epitopes. In this study we used a human Ad serotype 5 (Ad5) recombinant which expresses the full-length SIV $p55^{gag}$ protein of SIVmac251 from a DNA sequence inserted in the Ad E3 region (see Fig. 1) (Caravokyri *et al.*, 1993) to investigate the immune responses elicited when this vector was administered via different routes.

Methods

■ **Cell lines.** HeLa and 293 cells (Graham *et al.*, 1977) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS) and antibiotics. Mouse cell lines EL-4 and p815 were grown in RPMI 1640 containing 10% NCS, 20 mM HEPES and antibiotics, while L-cells and the baby hamster kidney cell line BHK-21 were grown in Glasgow modified Eagle's medium (GMEM) supplemented with 10% NCS and antibiotics. All cell lines were maintained in a 5% CO_2 incubator at 37 °C.

■ **Viruses.** The construction of Ad404p55 has been described previously (Caravokyri *et al.*, 1993). Briefly, the full length *gag* gene of SIVmac 251 was inserted in the E3 region of Ad5hr404 (Klessig & Grodzicker, 1979) and thereby placed under the control of the endogenous Ad major late promotor (MLP, Fig. 1). The hr404 mutation causes a single amino acid substitution in the E2A gene product (Kruijer *et al.*, 1981) and permits productive infection of simian cell cultures. Ad404p55 was propagated in HeLa cells and infectious particles were purified twice by CsCl density gradient centrifugation. The virus was then dialysed against 20% glycerol–PBS for 12 h to remove the CsCl and titred on 293 cells using a plaque assay before being aliquoted and stored at −70 °C. Ad327, which contains an E3 deletion identical to that of Ad404p55, was used as a control virus and purified similarly. Vaccinia virus expressing SIVp27 (vvp27) was isolated by M. Mackett, Patterson Institute, Manchester, UK and obtained from the MRC AIDS reagent programme (catalogue ADP260); it was grown and titred on BHK-21 cells.

■ **Animals and administration of viruses.** C57Bl/6 mice were purchased from Banton and Kingsman (Hull, UK) while BALB/c and C3H/He-mg animals were drawn from breeding colonies at the University of Warwick; they were used between 8 and 12 weeks of age. All experiments followed the guidelines laid down by the UK Co-ordinating Committee for Cancer Research. Mice were inoculated intraperitoneally with 10^8 p.f.u. of Ad404p55 or Ad327 in 200 µl PBS. For oral administration, 10^8 p.f.u. virus was again diluted in 200 µl PBS and delivered directly to the stomach using a gavage. Animals inoculated intranasally were anaesthetized with diethyl ether, inverted, and 15 µl of virus in PBS was placed in their nares for inhalation.

■ **ELISA.** Assays were performed in flat-bottom microtitre plates (Greiner, UK). Plates were coated with 50 µl of either purified SIV p27 (Almond *et al.*, 1990) at 2·0 µg/ml in distilled water (a generous gift of N. Almond, NIBSC, UK) or 1·0 µg/ml purified Ad327 and incubated overnight at 37 °C. Plates were washed in wash buffer (20 mM Tris pH 7·6, 200 mM NaCl, 0·1%, v/v, Tween 20) and wells blocked for 45 min with 5% BSA in wash buffer. Plasma samples were titrated as threefold serial dilutions and antibody was allowed to bind for 2 h. The wells were washed three times and biotinylated species-specific anti-mouse IgG, diluted 1:10000 from stock (Amersham), added for 90 min. IgA assays were similarly performed using a specific anti-mouse IgA reagent. After washing, 50 µl streptavidin–horse-radish peroxidase conjugate diluted 1:5000 from stock (Amersham) was added for 90 min and then 1,2-phenylenediamine dihydrochloride (Sigma) was added as substrate. Colour was allowed to develop and the reaction stopped by the addition of 50 µl per well of 2·5 M H_2SO_4. Absorbance at 492 nm was measured using a Labsystems Multiskan Plus ELISA plate reader (Life Sciences International). Antibody levels were determined by linear regression analysis of these data and values expressed as $\log_{10}$ endpoint titres. All assays included suitable controls to test the activity of the reagents.

■ **CTL assays.** Mice were sacrificed with CO_2. Spleens were removed immediately and splenocytes prepared by teasing through wire gauze. Splenocytes from naive mice were infected with Ad404p55 at a multiplicity of 5 p.f.u. per cell to provide stimulator cells. These were incubated with splenocytes from immunized mice (responders) at a ratio of 1:2 for 7 days in RPMI 1640 medium supplemented with 10% myoclone FCS (Gibco BRL), 0·05 mM β-mercaptoethanol and antibiotics to produce effector cells. Target cells were generated by infection of the appropriate major histocompatibility complex (MHC) class I-matched murine cell line (EL-4, p815 or L-cells), either with Ad327 at 48 h or with wild-type vaccinia virus or vvp27 at 24 h prior to the CTL assay and then loading with ^{51}Cr. Effector and target cells were co-cultured at ratios of 40:1, 20:1, 10:1 and 5:1 in RPMI 1640 (supplemented as above) for 4·5 h and the amount of total and released ^{51}Cr determined. The specific CTL activity in each case was expressed as % specific lysis, calculated using the following formula: released c.p.m. (test) − released c.p.m. (spontaneous)/released c.p.m. (total) − released c.p.m. (spontaneous).

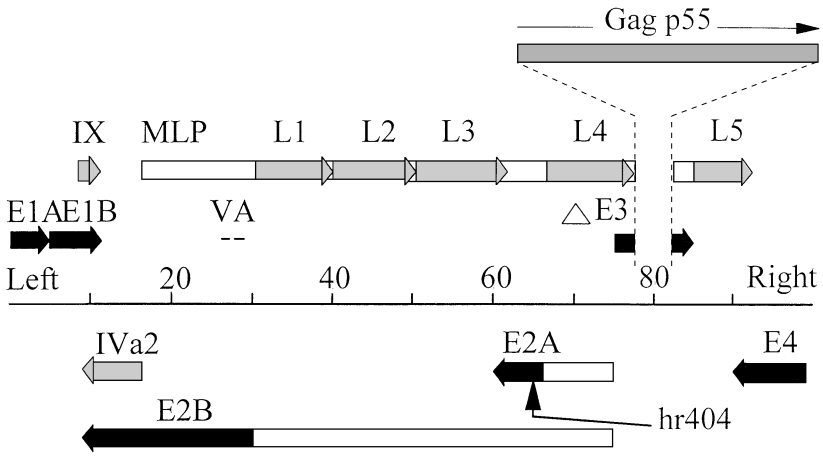

Fig. 1. Genome map of recombinant adenovirus Ad404p55. The linear genome is represented in the centre with rightwards transcripts above and leftwards transcripts below. Positions are indicated in map units; one map unit is equivalent to approximately 360 bp. Early genes are shown as black arrows and late genes as grey arrows; large introns are indicated by open boxes. The polIII transcripts VAI and II are shown as solid lines. The position of the hr404 point mutation is indicated by a bold arrow.

Results

Immune responses to Ad404p55 when administered intraperitoneally

The recombinant adenovirus Ad404p55 expresses the SIV Gag antigen, p55, from an endogenous Ad promotor (Caravokyri *et al.*, 1993). To determine whether Ad404p55 was capable of stimulating an immune response to SIV Gag, animals from three different strains of mice, each having a different MHC class I background, were immunized intraperitoneally with a single dose of Ad404p55. The induction of a humoral response (IgG) directed against the capsid antigen component, p27, of SIV $p55^{gag}$ was detected in all animals, with levels in C57Bl/6 (H-2^b) and C3H/He-mg (H-2^k) being significantly higher than those found in BALB/c (H-2^d) mice (Fig. 2).

To study further the effects of MHC haplotype on the response to p27, a CTL assay was established using EL-4, p815 and L-cells as MHC class I-matched targets for killing by effectors from C57Bl/6, BALB/c and C3H/He-mg mice respectively. These cell lines express MHC class I but not class II antigens. Significant levels of CTL activity directed against Ad antigens were identified in all three strains of mice (Fig. 3). However, CTLs against SIV p27 were only detected in C57Bl/6 and C3H/He-mg mice while in BALB/c animals only background levels of activity were observed. CTL activity was shown to be class I-restricted by performing a 'cross-over' experiment in which effector cells from each mouse strain were mixed with either class I-matched or unmatched target cells expressing p27. Only the class I-matched targets were lysed in each case (data not shown). These results indicate that there are epitopes on the SIV p27 antigen which are recognized in the context of H-2^b and H-2^k MHC class I antigens and which have the ability to stimulate specific CTL. Our assays would not detect any $CD4^+$ CTL activity which might also be stimulated by Ad404p27 infection. No antibody or CTL activity directed against SIV p27 was detected in animals given the Ad327 control virus (data not shown). Having identified

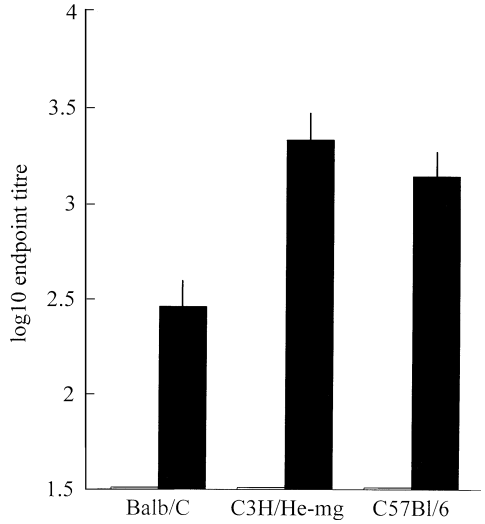

Fig. 2. Plasma anti-SIV $p27^{gag}$ IgG titres in BALB/c, C3H/He-mg and C57Bl/6 mice pre-immunization (open bars) and 4 weeks after intraperitoneal immunization (black bars) with 10^8 p.f.u. Ad404p55. Data shown are the mean determinations from *n* animals ± SD; BALB/c, $n = 8$; C3H/He-mg, $n = 3$; C57Bl/6, $n = 7$.

that C57Bl/6 mice were capable of making both a humoral and cellular response to SIV p27 expressed from Ad404p55, these animals were used in all further experiments.

Time-course of response after oral administration of Ad404p55

Oral administration is a simple route for vaccination of humans and is the established method of vaccination with adenovirus. We therefore wished to evaluate the nature of the response to Ad404p55 administered to mice in this way. After oral immunization with Ad404p55, mice were bled at various time-points and plasma samples assayed for IgG specific for either Ad antigens or SIV p27. In a series of experiments involving a total of 48 animals, three groups were identified depending on their humoral response: (1) 40% of experimental animals produced IgG to both Ad and SIV p27; (2) 16%

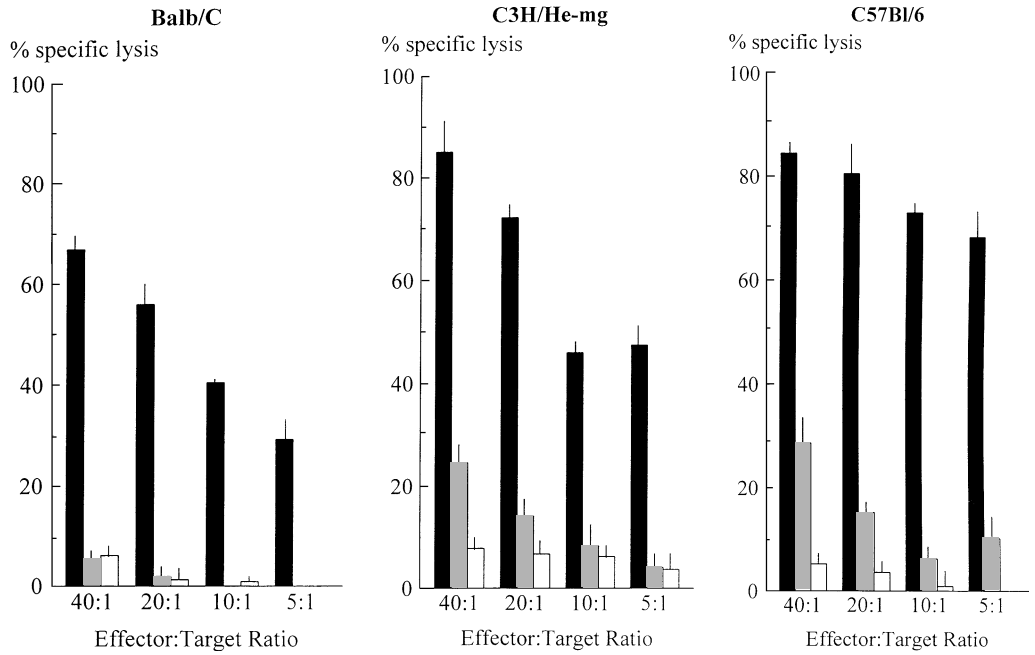

Fig. 3. Cytotoxic T cell responses in mice, 4 weeks after intraperitoneal immunization with Ad404p55. Splenocytes were taken from BALB/c, C3H/He-mg or C57Bl/6 animals, and assayed for CTL activity as described. Ad-specific CTL activity, black bars; vvp27-specific CTL activity, grey bars; vv-specific CTL activity, white bars. Data shown are means of three determinations ± SD.

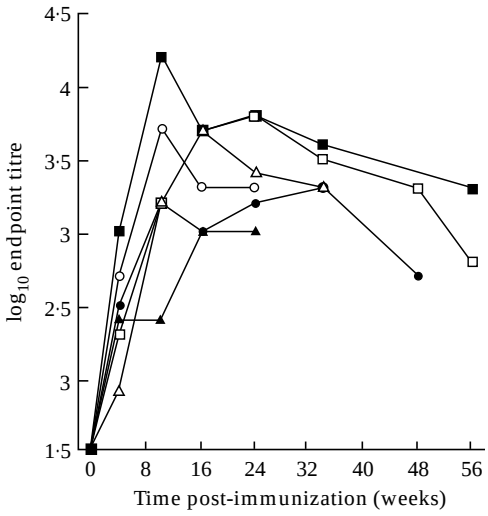

Fig. 4. Time-course of plasma anti-SIV p27^{gag} IgG titres in C57Bl/6 mice, immunized orally with 10^8 p.f.u. Ad404p55. The graph shows data from six individual animals; only a subset of animals showed a positive IgG response to p27 (see text).

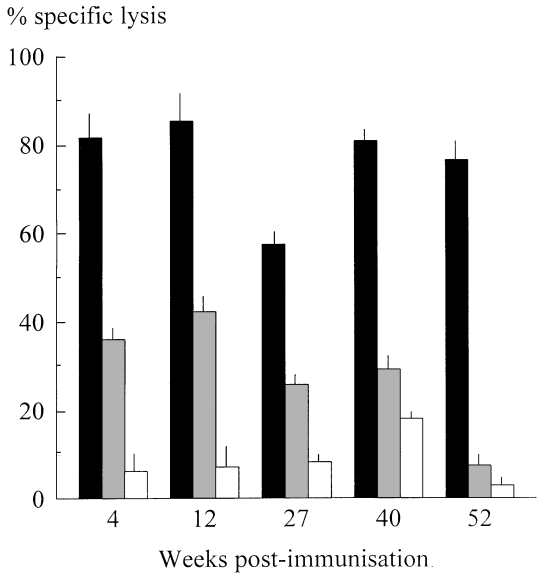

Fig. 5. Time-course of specific CTL activity in C57Bl/6 mice immunized orally with 10^8 p.f.u. Ad404p55 and selected for a positive anti-SIV p27^{gag} plasma antibody titre. Ad-specific CTL activity, black bars; vvp27-specific CTL activity, grey bars; vv-specific CTL activity, white bars. Data shown are for a 40:1 ET ratio and are means of three determinations ± SD.

produced IgG only to Ad antigens; and (3) 44% of animals failed to mount an IgG response to either antigen. Animals from this final group were not included in any studies as it was assumed they had not been infected by the inoculum.

The time-course of SIV p27-specific IgG levels in an experiment involving six responding animals is shown in Fig. 4. All animals had raised an IgG response to SIV p27 by 4 weeks post-immunization. These responses generally peaked at 8–12 weeks, with a mean titre of 3·3 $\log_{10}$, but still remained significant after 56 weeks. The CTL activity was also assessed over a 52 week period (Fig. 5). Animals maintained high levels of CTL activity against Ad antigens for the duration of the study. However, significant CTL activity against SIV p27 was only detectable for the first 27 weeks after immunization.

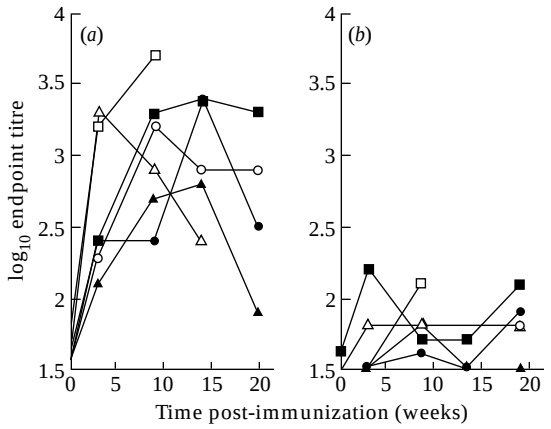

Fig. 6. Time-course of plasma anti-Ad (*a*) and anti-SIV p27^{gag} (*b*) IgG titres in C57Bl/6 mice immunized intranasally with 10^8 p.f.u. Ad404p55. The graphs show data from six individual animals.

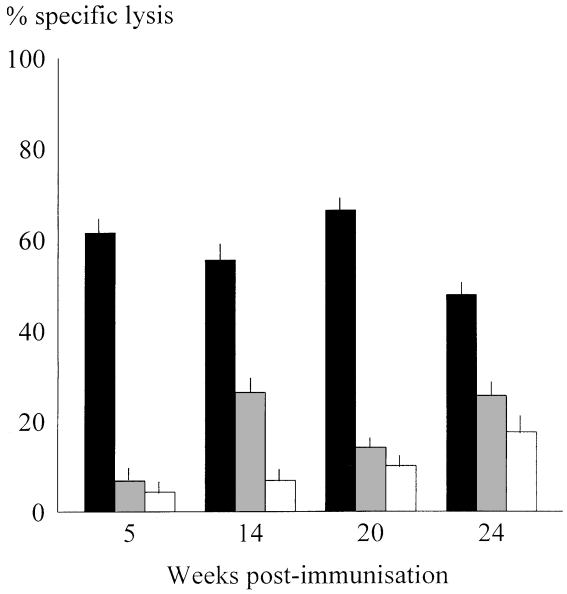

Fig. 7. Time-course of specific CTL activity in C57Bl/6 mice immunized intranasally with 10^8 p.f.u. Ad404p55. Ad-specific CTL activity, black bars; vvp27-specific CTL activity, grey bars; vv-specific CTL activity, white bars. Data shown are for a 40:1 ET ratio and are means of three determinations ± SD.

Intranasal administration of Ad404p55

As Ad are respiratory pathogens, intranasal administration was considered another suitable route through which an immune response to SIVp27 might be generated. In a series of animals immunized in this way, IgG specific for Ad antigens was readily detected in plasma indicating that the animals had been infected by the virus inoculum (Fig. 6*a*). However, when assays for SIV p27-specific IgG were performed, levels detected were low and transient and were not regarded as significant (Fig. 6*b*). When assays of specific CTL activity in these animals were performed (Fig. 7), a delayed CTL response was observed against both Ad antigens and SIV p27 which peaked at 14 weeks and then declined.

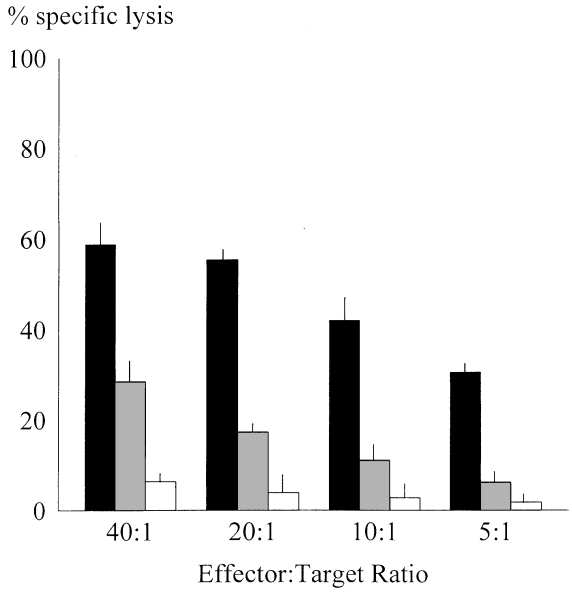

Fig. 8. CTL responses in a subset of C57Bl/6 mice, immunized orally with 10^8 p.f.u. Ad404p55, which showed no plasma SIV p27^{gag}-specific IgG response. Ad-specific CTL activity, black bars; vvp27-specific CTL activity, grey bars; vv-specific CTL activity, white bars. Data shown are means of three determinations, ± SD.

SIV p27-specific CTL activity in the absence of specific antibody

The pattern of response to SIV p27 in mice immunized intranasally with Ad404p55 was unexpected in that specific CTL activity was seen in the absence of significant plasma IgG activity. The pattern of specific antibody response observed in these mice broadly resembled that seen in those mice immunized orally with Ad405p55 that were described earlier as having a group 2 response. This finding led us to re-evaluate the significance of these different classes of plasma antibody response in animals immunized orally. Animals immunized orally with Ad404p55 and showing plasma IgG specific for Ad antigens but not for SIV p27 were therefore used as splenocyte donors for a CTL assay, 4 weeks post-immunization. Significant levels of specific CTLs were detected, not only for Ad antigens but also SIV p27 (Fig. 8). The levels of p27-specific CTL observed were similar to those seen in animals which also showed a strong humoral response to p27 (Fig. 5).

Given that both oral and intranasal immunization would be expected to stimulate local, mucosal immune responses, it was possible that the humoral response to SIV p27 in those animals showing no specific serum IgG was expressed purely as local IgA. To examine this possibility, extracts of faecal samples or intestinal washes taken at various times post-immunization from orally immunized animals and lung washings taken 4 weeks post-immunization from intranasally immunized animals were tested for specific IgA by ELISA. However, no activity was detected (data not shown).

Discussion

Although mice are considered as only semi-permissive for the replication of Ad5 vectors it has been shown that Ad constructs which are replication-competent in human cells can produce levels of expression from their foreign DNA insert sufficient to stimulate the immune system (Prevec *et al*., 1989). However, our observations show that there are considerable differences in the characteristics of the immune response elicited, depending on the route of administration. Immunization via the intraperitoneal route stimulated both plasma IgG production and specific CTL activity. However, this route is clearly unsuitable for larger animal models and man. Immunization through the oral route resulted in a Gag-specific response, but not all members of a group of animals could be infected reliably. Attempts to alter the 'take' of the vaccine by regulating the feeding regime of the animals before and after infection, or by neutralizing stomach acid with sodium bicarbonate at the time of infection, had no significant effect. The encapsulation of the vector (Lubeck *et al*., 1989) or, as has been described using replication-defective Ad recombinants, delivery in combination with an adjuvant (Ganne *et al*., 1994) may be ways of surmounting this problem.

A proportion of orally immunized animals failed to produce IgG directed against SIV p27, although they were capable of mounting a CTL response. Similarly, intranasal immunization resulted in a delayed CTL response, which was detected only 14 weeks post-immunization, and no p27-specific plasma IgG. It is possible that absence of a detectable humoral response in some animals merely reflects a lower efficiency of infection by the recombinant, with a resulting lower level of response to p27 that is below the level of detection by ELISA. However, the response to Ad antigens in these animals was not materially different in strength from that seen in animals making a full response to p27, suggesting that the efficiency of infection was similar. Also, the levels of SIV p27-specific CTL activity were similar in the two groups. Alternatively, the data may indicate that, under certain conditions, a pure cytotoxic response can be obtained. This finding is especially significant given the importance now attached to the CTL response in the control of HIV infection of humans (Rowland-Jones & McMichael, 1995). In the intranasally immunized animals, appearance of Gag-specific CTL activity was delayed. This might reflect the fact that the assays were not performed on immune cells derived from the primary site of immunization. Further work is needed to identify those factors which determine whether or not a humoral response to p27 is seen and to examine the levels of specifically reactive T cells in local lymphoid tissues in this system.

One of the potential advantages of recombinant Ad as a vaccine is that its route of infection is via mucosal surfaces, where it can stimulate local immune responses that could be of value in protecting against viruses such as HIV which also infect via this route. Having demonstrated that Ad404p55 could elicit both cellular and humoral systemic responses to p27 when delivered orally, we were unable to detect local IgA production in the gut despite repeated attempts. Efforts to identify specific IgA in the lungs of animals immunized intranasally were also unsuccessful. This was surprising as Ad vectors have been shown to stimulate IgA production previously (Lubeck *et al*., 1994; Mittal *et al*., 1995). It is possible that our assays were not sufficiently sensitive to detect low levels of IgA. However, this seems unlikely since they were performed according to protocols which detect specific IgA in faecal samples from mice immunized with other antigens (Z. Durrani, L. McLain & N. J. Dimmock, personal communication). It is also possible that the ability to stimulate an IgA response is itself dependent on the identity of the antigen; alternatively, the IgA response may be transient and may therefore have been missed in our assays.

The data presented here demonstrate that live recombinant Ad can stimulate long-lived cellular and humoral immune responses to an expressed antigen in mice. The antigen used for these studies, SIV p55^{gag} gives rise to the internal structural proteins of the virion and thus would not be expected to be a target for neutralizing antibody. Furthermore, single epitope Gag-specific CTL activity cannot prevent infection by SIV (Yasutomi *et al*., 1995), although responses to internal structural proteins are crucial to the resolution of infection by other viruses such as influenza virus (McMichael *et al*., 1986; Wraith *et al*., 1987). A pre-existing CTL response to SIV antigens may, however, serve to limit the virus load following subsequent infection, as indicated by the work of Bourgault *et al*. (1993), Gallimore *et al*. (1995) and Hu (1996). The properties of Ad404p55 reported here suggest that it merits further investigation as a vaccine in the SIV model system. It will therefore be of interest to examine the nature and extent of the mucosal response to this recombinant in more detail in both mice and macaques.

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