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Class I-restricted CTL induction by mucosal immunization with naked DNA encoding measles virus haemagglutinin

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We have investigated the class I-restricted CTL response specific for measles virus haemagglutinin (HA) in the spleens of mice immunized by various mucosal routes with a DNA plasmid carrying the HA gene (pV_1j-HA). A single immunization with recombinant DNA injected in the buccal mucosa induced an HA-specific CTL response. Similarly, nasal immunization with the DNA vaccine induced primary CTLs against measles virus HA. Booster immunization did not enhance the CTL activity. Oral or intrajejunal immunization with the plasmid induced a CTL response of lower magnitude. However, this could be potentiated by co-administration of the mucosal adjuvant cholera toxin or cationic lipids (DOTAP). These data show that a CTL response can be generated by mucosal vaccination using DNA vaccines.

Measles remains a major public health problem, causing the deaths of at least 1 million children annually in endemic regions. The lack of success of the present attenuated vaccine in developing countries is in part due to acquired maternal antibodies, which persist in infants until the age of 9 to 12 months (Albrecht *et al.*, 1977). Furthermore, the current live vaccine cannot be safely administered to immunocompromised children, and does not provide protection at mucosal surfaces which are known to be the portal of entry and primary site of replication of this virus (Walker, 1994).

DNA vaccination using recombinant plasmids encoding viral proteins is a promising alternative to the use of live vaccines. These vectors induce both humoral and cell-mediated immune responses to viral antigens in a way that mimics natural infection (Schödel *et al.*, 1994). Nucleic acid immunization was first reported to require targeting of the vector to long-lived cells such as myoblasts (Pertmer *et al.*, 1995; Ulmer *et al.*, 1993). However, more recent studies suggest that DNA vectors can provoke an immune response when delivered by a mucosal route, inducing humoral immunity (Fynan *et al.*, 1993; Hyde *et al.*, 1993; Ban *et al.*, 1996). Furthermore, targeting of the vector to professional antigen-presenting cells (APC), such as skin Langerhans cells, can be achieved by gene-gun inoculation, which enhances the immune response (Fynan *et al.*, 1993; Pertmer *et al.*, 1995).

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We examined the capacity of a plasmid (pV_1j-HA) encoding the haemagglutinin (HA) gene of measles virus to induce an HA-specific CTL response when administered by various mucosal routes. The plasmid was constructed by subcloning a cDNA encoding MV-HA (Gerald *et al.*, 1986) into the *Bgl*II site of plasmid pV_1j (Montgomery *et al.*, 1993), expression from which is driven by the CMV promoter. Site-directed mutagenesis was used to introduce *Bam*HI sites at the 5′ and the 3′ end of the MV-HA cDNA and to optimize the Kozak sequences of the ATGs (Kozak, 1986) in order to enhance expression of the encoded HA. The palindromic immunostimulatory sequences (Sato *et al.*, 1996) are present in pV_1j-HA.

We have previously reported that intramuscular immunization with the same vector induces HA-specific, Th1-biased humoral immunity and a class I-restricted specific CTL response (Cardoso *et al.*, 1996). In the present study, mucosal immunizations were performed with a single dose of 100 μg of pV_1j-HA, in BALB/c mice. This was given either by the nasal route (under light ether anaesthesia), the oral route (by gastric intubation) or the enteric route (by intrajejunal injection, under laparotomy). Since the buccal mucosa is covered by a network of dendritic cells analogous to skin Langerhans cells (Ahlfors & Czerkinsky, 1991), we also performed buccal immunizations, consisting of transepithelial injection of 100 μg of pV_1j-HA in the buccal mucosa.

Cholera toxin (CT) is a powerful mucosal adjuvant (Holmgren *et al.*, 1993). Its adjuvant effect on the HA-specific CTL response to pV_1j-HA was thus investigated by co-administration of the toxin with the vector. We also tested whether the anti-HA CTL response could be enhanced by addition of the cationic lipid DOTAP (Boehringer Mannheim) to pV_1j-HA prior to vaccination.

Mice were sacrificed 10 days after immunization, and the HA-specific CTL activity in their spleens was analysed as previously described (Beauverger *et al.*, 1993). Briefly, spleen

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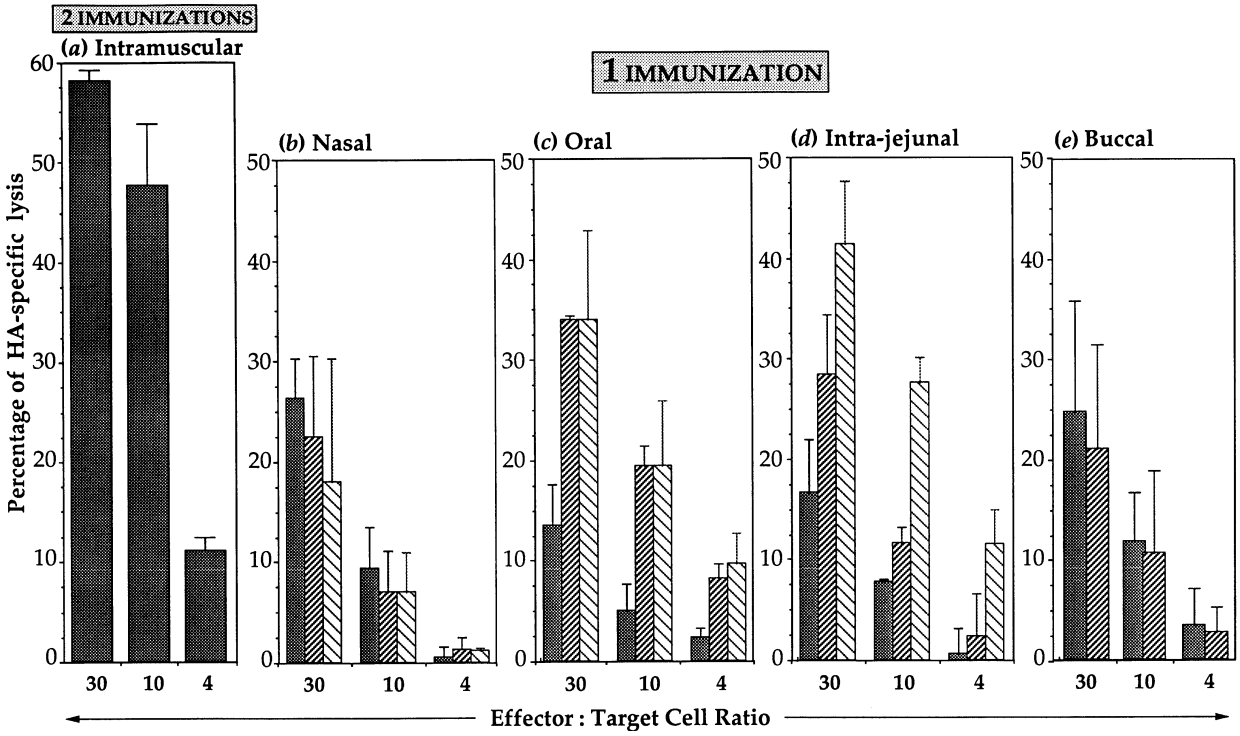

Fig. 1. Class I-restricted HA-specific CTL response induced in the spleens of BALB/c mice immunized once (*b*, *c*, *d*, *e*) or twice (*a*) with 100 μg of pV_1j-HA either alone (▩), with 5 μg CT (▨) or with 25 μg DOTAP (▧). Ten days after the last immunization, mice were sacrificed and spleen cells were restimulated for 7 days in bulk cultures with P815–HA transfectants. Cytotoxic activity was measured by ^{51}Cr release assay, using P815–HA and P815 cells as targets. The results are expressed as the percentage of HA-specific lysis, by subtracting the percentage (background) lysis of P815 target cells from the percentage lysis of P815–HA transfectant cells. The data are from one representative experiment out of three, using four mice per group. Background lysis never exceeded 20% of the positive control.

cells were co-cultured for 7 days with mitomycin-treated P815($H\text{-}2^d$)–HA stable transfectant cells, at a ratio of 10:1. Viable lymphocytes were then tested for cytolytic activity in a 5 h ^{51}Cr release assay using ^{51}Cr-labelled P815–HA target cells and untransfected P815 cells as a control, or class I-mismatched EL-4($H\text{-}2^b$)–HA transfectant cells. Results were expressed as percentage of HA-specific lysis calculated by subtracting the percentage lysis of untransfected P815 cells from that of P815–HA transfectant cells. Specific lysis was never observed on EL-4–HA cells, confirming that the cytolytic activity was restricted to class I molecules.

As a positive control, we gave mice two intramuscular injections of 100 μg of pV_1j-HA. This induced high levels of CTL activity in the spleen, reaching 60% of HA-specific lysis at an effector to target (E:T) ratio of 30:1 (Fig. 1*a*). In parallel, we examined the CTL response induced by pV_1j-HA administered by various mucosal routes. A single nasal immunization with 100 μg of pV_1j-HA induced a primary HA-specific CTL response in the spleen. The CTL activity reached 30% of specific lysis at an E:T ratio of 30:1 (Fig. 1*b*), and was not increased by further immunizations (Fig. 2). Co-administration of CT (5 μg) did not increase the CTL response. This result is in contrast with previous observations demonstrating an adjuvant effect of CT on the systemic CTL response after nasal immunization with a vaccinia–HA recombinant virus (Etchart *et al.*, 1996) and suggests that presentation of antigens expressed by DNA plasmids and viral recombinants may involve distinct pathways. Addition of DOTAP to the plasmid did not increase the CTL response. Although cationic lipids have been shown to enhance DNA uptake and transgene expression by epithelial cells of the lower respiratory tract (Hyde *et al.*, 1993), they did not increase influenza virus-specific antibody responses after nasal immunization with recombinant DNA (Ban *et al.*, 1996).

A single 100 μg dose of pV_1j-HA administered by the intragastric route induced a weaker splenic CTL response than obtained by the nasal route (Fig. 1*c*). However, contrary to observations made after nasal immunizations, co-administration of CT with pV_1j-HA intragastrically significantly enhanced the anti-HA CTL response ($P < 0{\cdot}01$, as calculated by Student's *t*-test, at an E:T ratio of 10:1). This increase was also observed when DOTAP was mixed with the plasmid prior to administration ($P < 0{\cdot}05$). Similar results were obtained after direct injection of pV_1j-HA into the gut lumen (intrajejunal immunization) (Fig. 1*d*). This response was also increased with CT ($P < 0{\cdot}05$), and even more so with DOTAP ($P < 0{\cdot}001$). The observation that similar levels of measles virus-specific CTLs are generated after either oral (intragastric) or intrajejunal

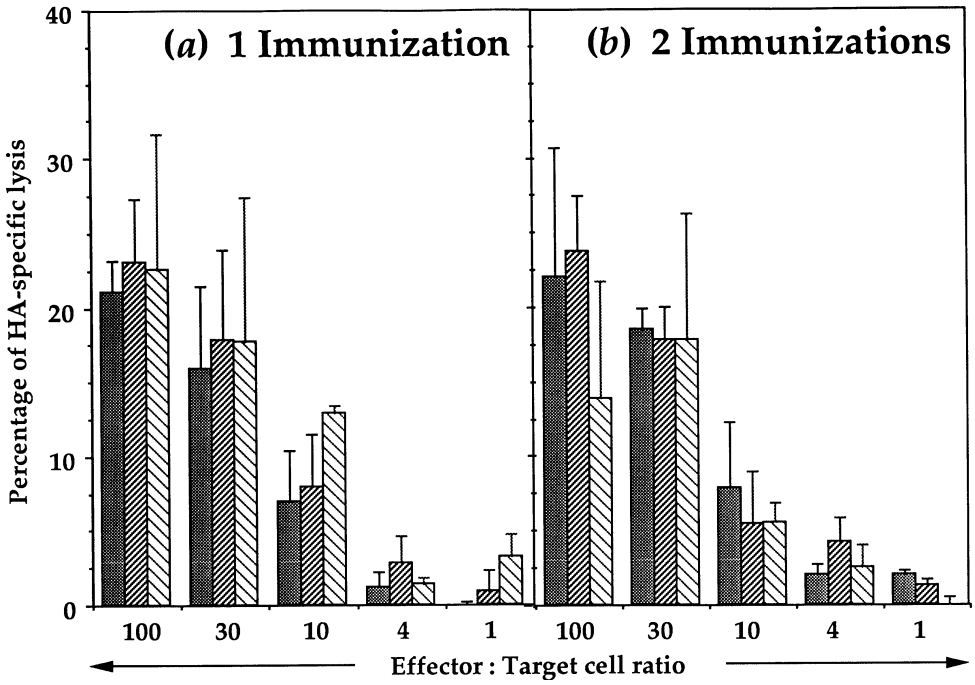

Fig. 2. HA-specific cytotoxicity in the spleens of mice immunized once (*a*), or twice within 21 days (*b*), by the nasal route with 100 μg of pV_1j-HA either alone (▩), with 5 μg CT (▨) or with 25 μg DOTAP (▧). Results are expressed as described in the legend to Fig. 1.

injection of naked DNA strongly supports the hypothesis that the DNA vector is not degraded in the stomach. Thus, DNA-based vectors may be efficient in oral immunization, compared with live virus vectors which are often highly sensitive to gastric degradation (Etchart *et al.*, 1996; Meitin *et al.*, 1994).

A single immunization with pV_1j-HA injected into the buccal mucosa also induced an anti-HA CTL response (Fig. 1*e*). This pertains to the presence within the buccal epithelium of functional antigen-presenting dendritic cells (Eriksson *et al.*, 1996). It should be noted that it is not possible to compare the efficiency of buccal immunization with 100 μg of plasmid injected into the buccal mucosa with the same dose delivered to the gut lumen or via the gastric route, where significant amounts of plasmid can be washed out. The observation that CT, co-injected with the plasmid via the buccal route, did not enhance the CTL response in the spleen may be attributed to its selective adjuvant effect on mucosa-associated lymphoid tissues (MALT) but not on pluristratified epithelia, such as that of the buccal mucosa.

Little is known concerning the APC type which captures and expresses DNA vaccines. DNA delivered through the skin (Hengge *et al.*, 1996) or by the nasal route (Hyde *et al.*, 1993) is transiently expressed by tissue-specific epithelial cells. However, studies on the intramuscular injection of recombinant DNA in bone-marrow chimeric mice demonstrated DNA expression by myoblasts but antigen presentation by donor type haematopoietic cells (Corr *et al.*, 1996; Ulmer *et al.*, 1996*a*, *b*). Several lines of evidence support the hypothesis that dendritic cells (DC) are involved in the presentation of proteins encoded by DNA vectors delivered by the mucosal route. DC capture antigens from epithelial tissues and migrate to draining lymph nodes for antigen presentation to T cells, *in vivo*. They also appear to act *in vitro* as potent APC for primary CTL induction after DNA transfection (Rouse *et al.*, 1994). Furthermore, the buccal mucosa contains a network of DC analogous to that of the skin Langerhans cells, and is an inductive site for T cell-mediated immune responses (Ahlfors & Czerkinsky, 1991). We have previously reported that buccal DC are endowed with APC function and the capacity to activate HA-specific $CD8^+$ T cells following local immunization with a viral peptide (Eriksson *et al.*, 1996). In addition, mucosae of the respiratory and gastro-intestinal tract also contain DC, which have been reported to act as efficient APC for mucosally delivered protein antigens (Liu & MacPherson, 1993; Holt *et al.*, 1994).

Taken together, these data show that mucosal immunization using a single dose of naked DNA is efficient for the induction of specific CTLs against measles virus, and presents a promising new approach for mass immunization against this virus.

References

Ahlfors, E. & Czerkinsky, C. (1991). Contact sensitivity in the murine oral mucosa. I. An experimental model of delayed-type hypersensivity reactions at mucosal surfaces. *Clinical and Experimental Immunology* **86**, 449–456.

Albrecht, P., Ennis, F. A., Saltzman, E. J. & Krugman, S. (1977). Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure. *Journal of Pediatrics* **91**, 715–718.

Ban, E. M., van Ginkel, F. W., Simecka, J. W., Curiel, D. T., Kiyono, H. & McGhee, J. R. (1996). Mucosal immunization with DNA encoding influenza A hemagglutinin (abstract). In *Nucleic Acid Vaccines for the Prevention of Infectious Diseases*. Bethesda: NIH.

Beauverger, P., Buckland, R. & Wild, T. F. (1993). Measles virus antigen induce both type-specific and canine distemper virus cross-reactive cytotoxic T lymphocytes in mice: localization of a common Ld-restricted nucleoprotein epitope. *Journal of General Virology* **74**, 2357–2363.

Cardoso, A., Blixenkröne-Moller, M., Fayolle, J., Liu, M., Buckland, R. & Wild, T. F. (1996). Immunization with plasmid DNA encoding for the measles virus hemagglutinin and nucleoprotein leads to humoral and cell mediated immunity. *Virology* **225**, 293–299.

Corr, M., Lee, D. J., Carson, D. A. & Tighe, H. (1996). Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *Journal of Experimental Medicine* **184**, 1555–1560.

Eriksson, K., Ahlfors, E., George-Chandy, A., Kaiserlian, D. & Czerkinsky, C. (1996). Antigen presentation in the murine oral epithelium. *Immunology* **88**, 147–152.

Etchart, N., Wild, F. & Kaiserlian, D. (1996). Mucosal and systemic immune responses to measles virus haemagglutinin in mice immunized with a recombinant vaccinia virus. *Journal of General Virology* **77**, 2471–2478.

Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C. & Robinson, H. L. (1993). DNA vaccines: prospective immunizations by parenteral, mucosal and gene-gun inoculations. *Proceedings of the National Academy of Sciences, USA* **90**, 11478–11482.

Gerald, C., Buckland, R., Barker, R. Freeman, G. & Wild, T. F. (1986). Measles virus haemagglutinin gene: cloning, complete nucleotide sequence analysis and expression in COS cells. *Journal of General Virology* **67**, 2695–2703.

Hengge, U. R., Chan, E. F., Walker, P. S. & Vogel, J. C. (1996). Cytokine gene expression in epidermis with biological effects following injection of naked DNA. *Nature Genetics* **10**, 161.

Holmgren, J., Lycke, N. & Czerkinsky, C. (1993). Cholera toxin and cholera toxin B-subunit as oral-mucosal adjuvant in antigen vector systems. *Vaccine* **11**, 1179–1184.

Holt, P. G., Haining, S. Nelson, D. J. & Sedgwick, J. D. (1994). Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. *Journal of Immunology* **153**, 256–261.

Hyde, S. C., Gill, D. R., Higgins, C. F., Trezise, A. E. O., MacVinish, L. J., Cuthbert, A. W., Ratcliff, R., Evans, M. J. & Colledge, W. H. (1993). Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy. *Nature* **362**, 250–255.

Kozak, M. (1986). Bifunctional messenger RNAs in eukaryotes. *Cell* **47**, 481–483.

Liu, L. M. & MacPherson, G. G. (1993). Antigen acquisition by dendritic cells: intestinal dendritic cells acquire antigen administered orally and can prime naive T cells in vivo. *Journal of Experimental Medicine* **177**, 1299–1307.

Meitin, C. A., Bender, B. S. & Small, P. A., Jr (1994). Enteric immunization of mice against influenza with recombinant vaccinia. *Proceedings of the National Academy of Sciences, USA* **91**, 11187–11191.

Montgomery, D. I., Shiver, J. W., Leander, K. R., Perry, H. C., Fiedman, A., Martinez, D., Ulmer, J. B., Donnelly, J. J. & Liu, M. A. (1993). Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. *DNA Cell Biology* **12**, 777–783.

Pertmer, T. M., Eisenbraun, M. D., McCabe, D., Prayaga, S. K., Fuller, D. H. & Haynes, J. R. (1995). Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine* **13**, 1427–1430.

Rouse, R. J. D., Nair, S. K., Lydy, S. L., Bowen, J. C. & Rouse, B. T. (1994). Induction in vitro of primary cytotoxic T-lymphocyte responses with DNA encoding herpes simplex virus proteins. *Journal of Virology* **68**, 5685.

Sato, Y., Roman, M., Tighe, H., Lee, D., Corr, M., Nguyen, M. D., Silverman, G. J., Lotz, M., Carson, D. A. & Raz, E. (1996). Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354.

Schödel, F., Aguado, M.-T. & Lambert, P.-H. (1994). Introduction. In *Nucleic Acid Vaccines*, WHO, Geneva. *Vaccine* **12**, 1491–1492.

Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. M., Friedman, A., Hawe, L. A., Leander, K. R., Martinez, D., Perry, H. C., Shiver, J. W., Montgomery, D. L. & Liu, M. A. (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**, 1745–1749.

Ulmer, J. B., Deck, R. R., DeWitt, C. M., Donnelly, J. J. & Liu, M. A. (1996*a*). Generation of MHC class I-restricted cytotoxic T lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells. *Immunology* **89**, 59–67.

Ulmer, J. B., Shiver, J. W., Caulfield, M., Liu, M. A. & Donnelly, J. J. (1996*b*). Mechanisms of immunity and antigen presentation with DNA vaccines (abstract). In *Nucleic Acid Vaccines for the Prevention of Infectious Diseases*. Bethesda: NIH.

Walker, R. I. (1994). New strategies for using mucosal vaccination to achieve more effective immunization. *Vaccine* **12**, 387–400.

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