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Immunology

Immunological properties of hepatitis B core antigen fusion proteins

(peptide/virus/vaccine)

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ABSTRACT **The immunogenicity of a 19 amino acid peptide from foot-and-mouth disease virus has previously been shown to approach that of the inactivated virus from which it was derived after multimeric particulate presentation as an N-terminal fusion with hepatitis B core antigen. In this report we demonstrate that rhinovirus peptide–hepatitis B core antigen fusion proteins are 10-fold more immunogenic than peptide coupled to keyhole limpet hemocyanin and 100-fold more immunogenic than uncoupled peptide with an added helper T-cell epitope. The fusion proteins can be readily administered without adjuvant or with adjuvants acceptable for human and veterinary application and can elicit a response after nasal or oral dosing. The fusion proteins can also act as T-cell-independent antigens. These properties provide further support for their suitability as presentation systems for "foreign" epitopes in the development of vaccines.**

An important consideration for designing vaccines based on defined peptides from complex protein antigens is their optimal delivery to the immune system to produce maximal antibody response, both qualitatively and quantitatively. This requirement has resulted in the development of various polymeric presentation systems based on recombinant DNA technology—for example, core- (1, 2) and surface-antigen (3) particles from hepatitis B virus, poliovirus particles (4), and yeast transposon Ty proteins (5).

The use of the core antigen from hepatitis B virus (HBcAg) to present "foreign" peptide epitopes, first described by Newton *et al.* (6) in 1986, offers several potential advantages. The protein subunit is a 21-kDa polypeptide (7, 8) that spontaneously assembles into characteristic 27-nm particles (9) and can be expressed in a wide range of systems, including bacterial cell (8), yeast cell (10), and mammalian cell (11), or via vaccinia virus (1, 6), and baculovirus (12). The core particle is known to be highly immunogenic (13), possibly due to its polymeric nature, the presence of a number of well-defined helper T-cell epitopes (14), and its ability to function as a T-cell independent antigen (15). Furthermore, N-terminal (1, 6) and C-terminal (2) fusions plus insertions into surface loop structures (B.E.C., A.L.B., and M.J.F., unpublished data) have all been shown to produce chimeric core particles. A study using foot-and-mouth disease virus peptide sequence fused to the N terminus of HBcAg and expressed via vaccinia virus demonstrated that the immunogenicity could approach that of inactivated foot-and-mouth disease virus particles (1). However, the poor yield in the vaccinia virus expression system provided insufficient material for detailed immunological investigations. We have now extended our studies of the HBcAg presentation system to make use of bacterial (16) and yeast (K. M. Beesley, M.J.F., B.E.C., and M. A. Romanos, unpublished data) expression vectors to enable us to carry out a more thorough examination of the immunological properties of these fusion proteins.

In this paper we present data on the immunogenicity of particles composed of a peptide from the VP2 protein of human rhinovirus type 2 (HRV2) (17) fused to the N terminus of HBcAg that have been expressed in *Escherichia coli* cells (16). Activity of the product is compared with that of the peptide when it is presented either alone or covalently linked to keyhole limpet hemocyanin (KLH). The requirement for adjuvants, topical routes of administration, and T-cell independent responses are also described.

MATERIALS AND METHODS

Animal Inoculations. Female Dunkin–Hartley guinea pigs (Harlan Porcellus, Heathfield, Sussex, U.K.) weighing ≈400 g and female mice (Harlan Olac, Bicester, Oxon, U.K.) ≈8 weeks old were used for immunogenicity studies. Further details of antigen doses, inoculation schedules, and sampling for individual experiments are given in *Results*.

Synthetic Peptides. Sequences corresponding to HRV2 VP2 156–170 (17) and 156–170 plus a helper T-cell epitope from VP2 24–33 (18) were synthesized using an adaptation of the Merrifield technique (19) described by Houghten (20). Each peptide had an additional nonnatural cysteine residue at its carboxyl terminus to facilitate coupling to KLH via an *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (21).

Construction of Recombinant Plasmids and Production of Fusion Proteins. The system used was an engineered plasmid vector pBC404 (pAT 153 based) with gene expression being driven by the *tac* promoter (22). Basically the plasmid was engineered so that RNA transcripts produced from the *tac* promoter initiated translation at an AUG codon adjacent to a unique *Eco*RI restriction site. A second unique site, *Bam*HI, was inserted upstream of a gene coding for HBcAg that had been isolated from CsCl-purified Dane particles (adw serotype) (16). This *Bam*HI linker is located within the precore signal sequence that normally leads to secretion of the core protein from the cell. To construct chimeric fusion particles coding for a specific N-terminal HRV2 VP2 epitope, synthetic oligonucleotides with cohesive ends for *Eco*RI (5′) and *Bam*HI (3′), respectively, were prepared using an Applied Biosystems 381A DNA synthesizer. These oligonucleotides were ligated into *Eco*RI/*Bam*HI-digested pBC404 that had been purified from 1% low-melting-point agarose by standard methods (16). This DNA was then transformed into *E. coli* strain JM101, and recombinant plasmids were restriction mapped from small-scale DNA preparations.

Bacteria harboring recombinant plasmids were grown overnight in L-broth/ampicillin medium to high cell density

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Abbreviations: HBcAg, hepatitis B core antigen; IFA, incomplete Freund's adjuvant; HRV2, human rhinovirus type 2; KLH, keyhole limpet hemocyanin.

and diluted with fresh L-broth (1:10) the following day. Expression was routinely induced by the immediate addition of isopropyl thiogalactoside (60 μg/ml, final concentration), and the bacteria were allowed to replicate for a further 6–8 hr at 37°C. Bacteria were then harvested, and chimeric core particles, with N-terminal peptide epitopes, were purified and characterized as described (1, 16).

Enzyme-Linked Immunosorbent Assay (ELISA). Anti-HRV2 peptide and anti-HBcAg activities in serum samples were measured by using a modification of the indirect or double-antibody sandwich ELISA methods (23). In the sandwich ELISA human anti-HBcAg antibody (1:200) was used to trap HBcAg at 2 μg/ml onto the plate, whereas in the indirect ELISA peptide at 2 μg/ml was coated directly onto the plastic. The plates were then washed and test serum samples at a series of 0.5 logarithmic (to base 10) dilutions from 10^{-1} were added. After 1-hr incubation at 37°C the plates were washed, and anti-guinea pig IgG–peroxidase conjugate was added. After a further hour at 37°C the plates were washed, and an enzyme substrate (0.04% *o*-phenylenediamine plus 0.004% hydrogen peroxide in phosphate/citrate buffer) was added. The resulting color development was stopped with 12.5% (vol/vol) sulfuric acid after 5–7 min, and the absorbance at 492 nm was measured in a Titertek Multiskan reader (Flow Laboratories).

The A_{492} values obtained from dilutions of postinoculation samples were plotted against the logarithmic (to base 10) reciprocal antiserum dilution, and the antibody titer was calculated by reference to a negative standard (1:10 dilution of preinoculation serum).

RESULTS

Comparative Immunogenicity of HBcAg Fusion Protein with KLH-Linked and Uncoupled Peptides. The immunogenicity of the HRV2 peptide–HBcAg fusion protein was compared with synthetic HRV2 peptide coupled to KLH and to uncoupled HRV2 peptide with an added helper T-cell epitope by inoculating groups of four guinea pigs i.m. with each preparation at a range of HRV2 peptide doses from 150 μg to 0.015 μg in incomplete Freund's adjuvant (IFA). All animals were also reinoculated with the same material at 56 days, and samples were collected 28, 56, and 70 days after the primary immunization and analyzed for anti-HRV2 peptide activity (Fig. 1).

HRV2 peptide antibodies were elicited by all three preparations at 28 days, and higher levels were found at 56 days. In terms of comparative immunogenicity, on a weight-for-weight basis, 150 μg of uncoupled peptide, 15 μg of KLH-coupled peptide, and 1.5 μg of peptide in the form of HBcAg fusion protein were required to elicit ≈3 logarithmic (to base 10) units of anti-HRV2 peptide antibody after a single inoculation. The HBcAg fusion protein also elicited a primary antibody response against a smaller (0.15 μg) dose of peptide. After reinoculation at 56 days, increased antipeptide responses to all three preparations were seen, with the KLH-coupled material showing the greatest (≈10-fold) improvement. However, the response to the HBcAg fusion protein was still superior in terms of antipeptide titer.

Requirement for Adjuvant in the Immune Response to HBcAg Fusion Protein. The effect of adjuvant on the immune response to the HBcAg fusion protein was studied by inoculating groups of four guinea pigs or eight BALB/c mice i.m. with 20-μg doses of fusion protein, incorporating 1.5 μg of HRV2 peptide, added to IFA, 0.4% aluminum hydroxide (20% "Alhydrogel"), or phosphate-buffered saline with no adjuvant. All animals were also reinoculated with the same material at 84 days to study the memory response.

The anti-HBcAg response over the first 28 days and anti-HRV2 peptide response over the first 14 days were similar in both species receiving IFA or aluminum hydroxide

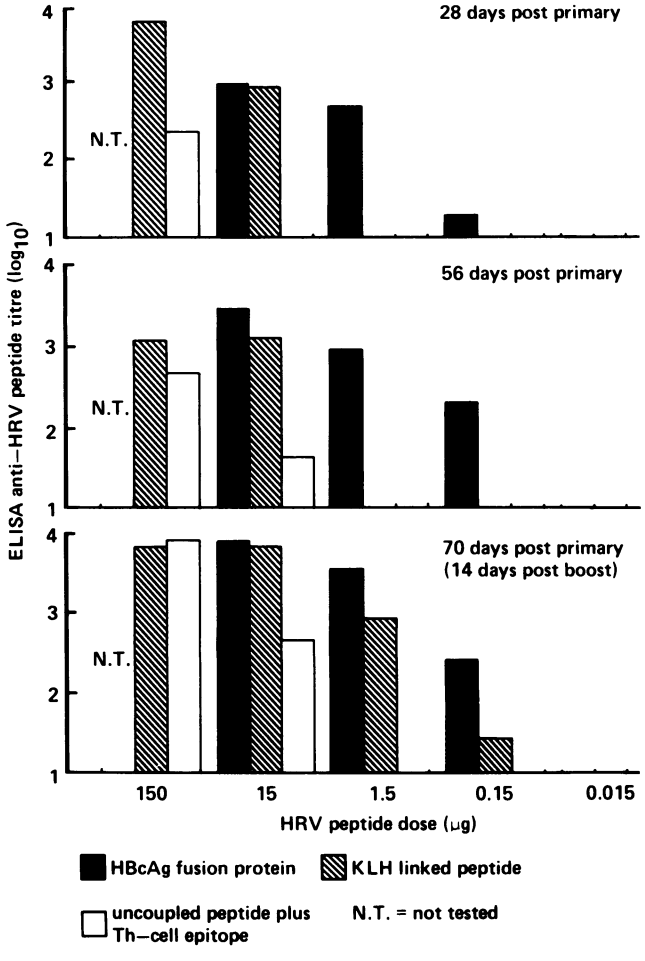

FIG. 1. Comparative immunogenicity of HBcAg fusion protein, KLH-coupled HRV2 peptide, and uncoupled HRV2 peptide in guinea pigs. Data were obtained at 28, 56, and 70 days after primary immunization; animals were boosted with a second dose at 56 days. Columns represent the levels of anti-HRV2 peptide antibody elicited by the three preparations.

adjuvant preparations (Figs. 2 and 3). After this time the response to the oil emulsion preparation tended to be greater and to persist at higher levels up to the 84-day boost. The response to nonadjuvanted fusion protein was ≈10-fold lower than the responses to the adjuvanted preparations, but, nevertheless, significant levels of anti-HBcAg and anti-HRV2 peptide antibody were produced. After a single reinoculation at 84 days, antibody levels in the IFA and aluminum hydroxide groups were boosted to similar levels within 7 days. The nonadjuvanted groups also displayed a marked anamnestic response, such that the levels approached those of the postboost adjuvanted preparations in the BALB/c mice (Fig. 3) and were equivalent to primary adjuvanted responses in guinea pigs (Fig. 2).

Nasal and Oral Immunization with HBcAg Fusion Protein. To determine whether it was possible to immunize animals by topical nasal and oral administration of the HBcAg fusion protein, three groups of six BALB/c mice were given 20 μg of fusion protein, with no added adjuvant, either intranasally, orally, or i.m. Each group of animals also received further doses by the same route 28 and 56 days later. Serum samples collected at 0, 28, 56, and 84 days were analyzed for anti-HBcAg and anti-HRV2 peptide antibody.

After two intranasal inoculations of fusion protein anti-HBcAg [1.8 logarithmic (to base 10) units] and to a lesser extent anti-HRV2 peptide [1.1 logarithmic (to base 10) units] antibodies were detected in the serum (Fig. 4). These levels

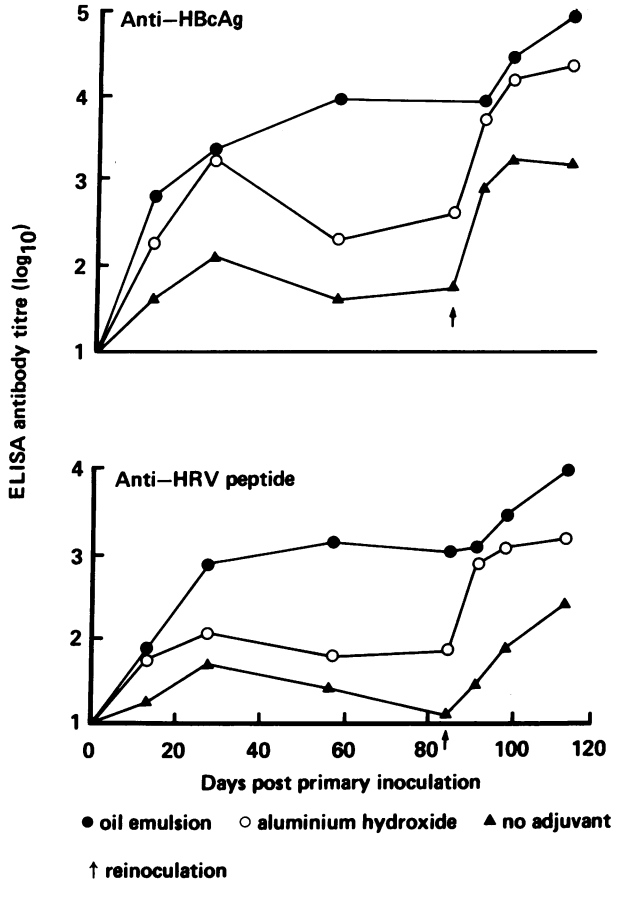

FIG. 2. Effect of adjuvant on the immune response of guinea pigs to HBcAg fusion protein. Sera collected at 14- to 28-day intervals for 84 days after primary inoculation and at 7- to 28-day intervals after reinoculation with HBcAg fusion protein in an IFA oil emulsion, in aluminum hydroxide, or with no adjuvant have been analyzed for anti-HBcAg and anti-rhinovirus peptide activity.

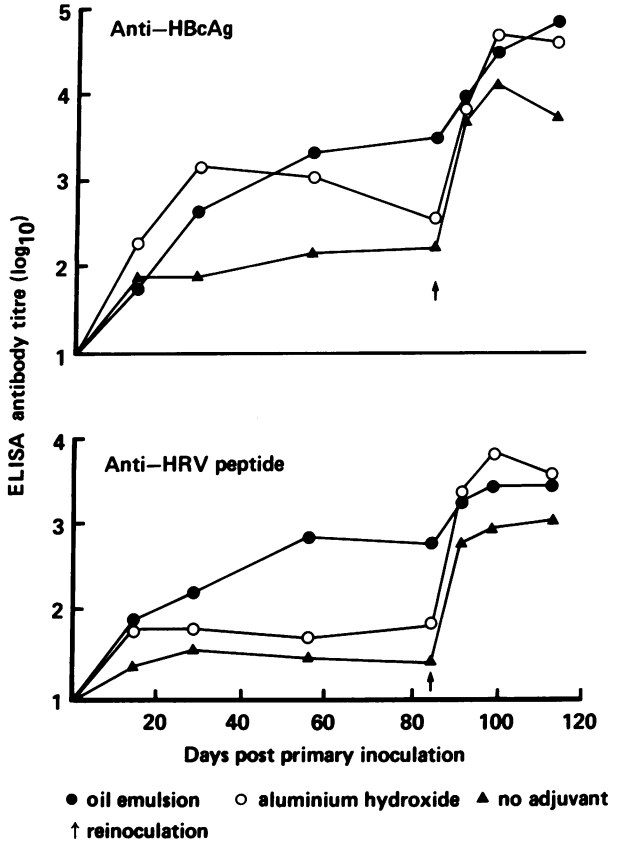

FIG. 3. Effect of adjuvant on the immune response of BALB/c mice to HBcAg fusion protein. Sera collected at 14- to 28-day intervals for 84 days after primary inoculation and at 7- to 28-day intervals after reinoculation with HBcAg fusion protein in an IFA oil emulsion, aluminum hydroxide, or with no adjuvant have been analyzed for anti-HBcAg and antirhinovirus peptide activity.

were further increased after a third intranasal dose at 56 days. A single oral dose of fusion protein also elicited low levels of anti-HBcAg [1.7 logarithmic (to base 10) units] and anti-HRV2 peptide [1.1 logarithmic (to base 10) units] in the serum. These levels persisted for 84 days but did not appear to be significantly altered by the second and third inoculations. In comparison, i.m. inoculation of the nonadjuvanted fusion protein produced significant levels of anti-HBcAg and anti-HRV2 peptide after one inoculation, and these levels were further boosted by subsequent inoculations to titers of 4.3 logarithmic (to base 10) units for anti-HBcAg and 2.3 logarithmic (to base 10) units for anti-HRV2 peptide (Fig. 4). Analysis of antibody classes in the serum revealed that the dominant response was IgG followed by IgA and then IgM after all three routes of administration. However, the IgG/IgA ratio after nasal or oral dosing was 6–10:1, whereas after i.m. inoculation the ratio was 370:1, suggesting marked variation in distribution of antibody classes in the serum after the different routes of administration.

T-Cell Independent Response to HBcAg Fusion Protein. The T-cell dependence of the immune response to HBcAg was examined by inoculating two groups of eight outbred MF1 euthymic or MF1-nu/nu athymic mice i.m. with 20 μg of fusion protein in IFA and reinoculating at 56 days. Serum samples collected at regular 7- to 14-day intervals were analyzed for anti-HBcAg and anti-HRV2 peptide activity.

The athymic mice produced low but significant levels of anti-HBcAg and anti-HRV2 peptide antibodies after a single inoculation of fusion protein (Fig. 5). However, these levels were ≈100-fold lower than those seen in euthymic mice. Reinoculation at 56 days resulted in a small increase in anti-HBcAg and anti-HRV2 peptide levels in the euthymic

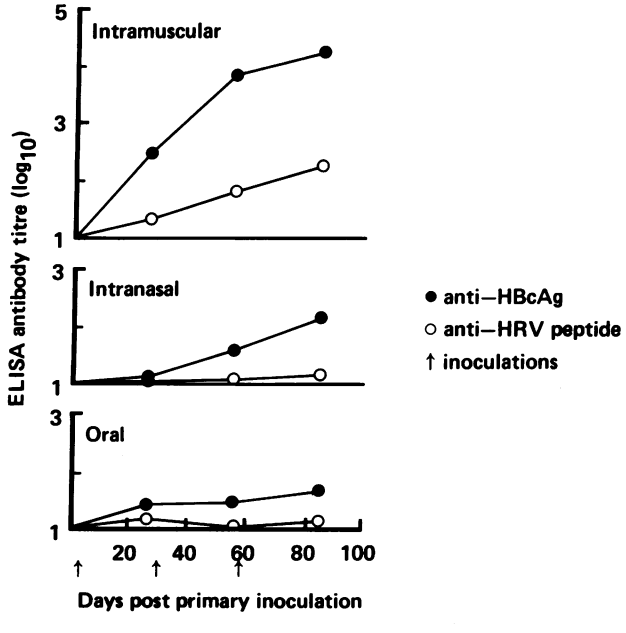

FIG. 4. Serum antibody response of BALB/c mice to HBcAg fusion protein administered by i.m., nasal, or oral routes. Sera collected at the time of three separate monthly inoculations and at 1 mo afterwards have been analyzed for anti-HBcAg and anti-rhinovirus peptide activity.

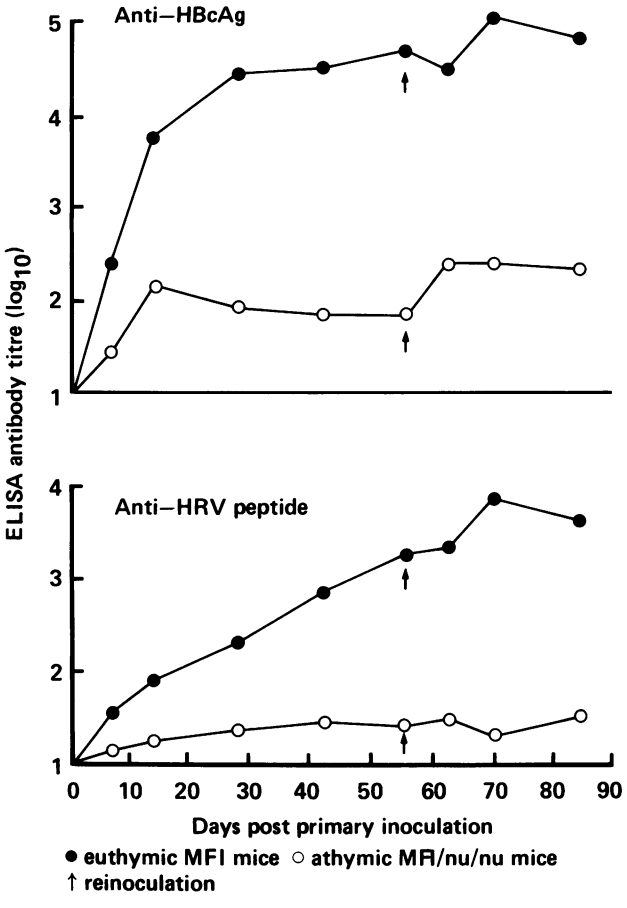

FIG. 5. Thymic dependent and independent response of MF1 mice to HBcAg fusion protein. Sera collected at 7- to 28-day intervals after primary inoculation and reinoculation at 56 days in euthymic and athymic mice have been analyzed for anti-HBcAg and anti-rhinovirus peptide antibody responses.

mice but increased only anti-HBcAg levels in athymic mice. The response was no greater than that seen after primary inoculation (Fig. 5).

DISCUSSION

Recent publications on a number of polymeric peptide delivery systems based on self-assembling particles (1–6) have stimulated considerable interest within the field of vaccine development. However, it is important to demonstrate in comparative experiments that these systems have direct advantages over more traditional peptide/carrier approaches.

In this paper we present studies on the immunological properties of chimeric particles of HBcAg with a short peptide from HRV2 VP2 (17) fused to its N-terminus. Such core fusion proteins have already been shown to depend on both their particulate nature and their helper T-cell activity for their inherent immunogenicity (24). These properties now appear to make them at least 100-fold more immunogenic than an uncoupled peptide with a single added helper T-cell site and 10-fold more immunogenic than a synthetic peptide chemically coupled to KLH by a C-terminal cysteine residue.

Another important consideration is the requirement for adjuvants to achieve acceptable immunogenicity. In a number of previous publications (2, 3, 5, 15, 25) claims on immunogenic activity have been based on multiple inoculations of laboratory animals when using complete Freund's adjuvant. Although such an adjuvant is valuable for assessing whether a product is immunogenic, the fact that it is unacceptable for use in either domestic animals or humans means that the use of other adjuvants is required. In this study we have compared the immunogenicity of HBcAg fusion proteins in IFA—currently used in some veterinary vaccines—in aluminum hydroxide—acceptable for use as an adjuvant in humans—and in the absence of adjuvant. Although IFA gives the greatest response after primary immunization, aluminum hydroxide is a suitable adjuvant after single reinoculation. Furthermore, the fusion protein is also immunogenic in the absence of adjuvant and primes for a significant anamnestic response. Therefore, the choice of adjuvant should not present problems for the practical application of HBcAg fusion protein-based vaccines.

Two other goals of many innovative vaccine preparations are to provide for easier administration by overcoming the requirement for needle inoculation and to induce local secretory immunity. Because it has been reported (26) that particulate antigens may elicit an immune response after local administration we were interested to see whether this situation would apply to HBcAg fusion proteins. Our studies show that HBcAg fusion proteins will elicit a serum antibody response against HBcAg and the fused peptide sequence after nasal or oral administration, although actual levels of antibody are low. Nevertheless, the modified antibody class distribution indicates that a secretory response is induced, which may, in turn, indicate priming of this important arm of the immune system for a subsequent encounter with the pathogen by parenteral inoculation with the vaccine (27).

Finally, reports that HBcAg can function as a T-cell-independent antigen (15, 25) present the possibility of immunizing immunodeficient patients with HBcAg fusion proteins. In this study we have demonstrated anti-HBcAg and anti-HRV2 peptide antibody responses in T-cell-deficient outbred nude mice, although the immunogenicity is substantially poorer than that seen in euthymic mice. Furthermore, the T-cell-independent anti-HBcAg response is lower than that reported by Milich and coworkers (15, 25), possibly due to the choice of adjuvants—IFA in our study and complete Freund's adjuvant in previous reports. Nevertheless, the ability of these fusion proteins to produce a T-cell-independent antibody response against a short peptide antigen may offer a means of actively immunizing immunocompromised patients and thus provide prophylaxis for individuals on immunosuppressive drugs or immunotherapy for patients with acute acquired immunodeficiency syndrome (AIDS).

It is interesting to note that antipeptide titers elicited by the added HRV2 sequence were, in general, very similar to antivirus titers observed in the sera by indirect ELISA. Furthermore, in samples with high levels of antibody against the peptide [>3.5 logarithmic (to base 10) units] some low levels of neutralizing activity [up to 1.7 logarithmic (to base 10) units of neutralization at a 50% endpoint against 100 tissue culture 50% infective dose of virus] were seen. However, the ratio of antipeptide antibody to virus neutralizing antibody of ≈100:1 was no better than that seen previously (17) with KLH-coupled synthetic HRV2 peptide. Therefore the N-terminal fusion of this peptide to HBcAg apparently does not present the HRV2 peptide in a conformation favorable for the production of greater levels of neutralizing antibody. This interpretation has led us to examine the possibility of inserting the peptide into defined immunogenic sites on the HBcAg particle (B.E.C., A.L.B., and M.J.F., unpublished data) in the hope that the secondary structure will then be more representative of the form that it adopts on the virus particle and that the enhanced response against core antigen seen in all our experiments can then be directed toward the inserted foreign peptide.

In conclusion, we have demonstrated improved immunogenicity of HBcAg fusion proteins over that of KLH-coupled

or uncoupled synthetic peptide, activity in the absence of adjuvants or in adjuvants acceptable for human and veterinary use, ability to immunize by nasal or oral routes, and a T-cell-independent response. These features provide further evidence on the suitability of the HBcAg presentation system for vaccines. Furthermore, the fact that N- (1, 6) and C-terminal (2) fusions can be tolerated as well as insertions into predicted surface loop structures (B.E.C., A.L.B., and M.J.F., unpublished data) without affecting the self-assembly properties offers an opportunity to study the effect of peptide location and structural restriction on immunogenicity. These results should also provide standards against which other particulate presentation systems may be judged.

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