Heterotypic recognition of foot-and-mouth disease virus by cattle lymphocytes

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Lymphoproliferation against foot-and-mouth disease (FMD) virus was examined using peripheral blood mononuclear cells from vaccinated cattle. Ten weeks after revaccination the optimum conditions for proliferation were obtained with 1 µg/ml of purified virus after 5 to 6 days in culture. This contrasted with the response at 20 months post-revaccination, when the response required less antigen and showed a peak response after 3 to 4 days in culture. Proliferation was specific for FMD virus, but was cross-reactive between serotypically distinct strains of the virus. The proliferative response to isolated virus proteins (VP) involved all three major capsid proteins (VP1, -2 and -3), although the proliferation of lymphocytes from heterotypically vaccinated cattle was due to VP3. Furthermore, the response induced by purified virus, chemically fixed virus and subunit virus particles was indistinguishable and thus it is likely that processing was required for the induction of proliferation. Together these data strongly suggest that FMD virus-induced lymphoproliferation is T cell-mediated and that VP3 may contain dominant, cross-reactive sequences.

Introduction

Foot-and-mouth (FMD) is a highly contagious disease of cloven-hoofed animals and is of great importance to the agricultural communities of the world. The aetiological agent for FMD is a picornavirus (FMDV) and is composed of 60 copies each of four structural proteins (VP1, -2, -3 and -4) that enclose a positive sense ssRNA genome. The intact virus particle (146S) is heat-labile (Ahl, 1968; Doel & Baccarini, 1981) and pH-labile (Randrup, 1954; Asso et al., 1966) and may be disrupted to form a range of subunit particles which include a 12S pentamer of VP1, -2 and -3 (Vasquez et al., 1979). All three proteins contribute to antigenic determinants on the surface of the virus, but only isolated VP1 has been shown to induce neutralizing antibody (Laporte et al., 1973; Bachrach et al., 1975). In many countries, the control of FMD is by the use of inactivated virus vaccine. These vaccines suffer from a variety of disadvantages, including thermal instability and frequent association with field outbreaks of disease (King et al., 1981). Consequently, considerable effort has been made to develop synthetic alternatives to conventional FMD vaccines.

Immunization with VP1 derived either from virus or as a recombinant protein expressed in Escherichia coli cells (Kleid et al., 1981) was found to induce poor immunity. However, two regions of VP1 were identified which were capable of inducing neutralizing antibody (Strohmaier et al., 1982) and, subsequently, synthetic peptides representing either or both of the two regions (residues 141 to 160 and 200 to 213) have been used to investigate the potential of peptides as vaccine antigens (Bittle et al., 1982; Francis et al., 1985; DiMarchi et al., 1986; Murdin & Doel, 1987a, b).

Most of the studies in our laboratory have been with a synthetic peptide based on residues 200 to 213 and 141 to 158 of VP1 of the O1 Kaufbeuren strain of FMDV joined by a -Pro-Pro-Ser- sequence and terminated at one or both ends by a non-native cysteine residue (DiMarchi et al., 1986). The ability of this antigen to induce both peptide- and virus-specific antibodies clearly indicates that it is immunogenic with respect to cattle B cells. The peptide also induces protective immunity in cattle, although the titres of neutralizing antibody obtained do not confer the level of protection that would be anticipated by comparison with virus vaccination (Pay & Hingley, 1987). Thus, it is likely that the peptide-specific antibodies are qualitatively different from those induced by whole virus and may reflect inappropriate T cell help.

Although nothing has been published about the cattle T cell response to peptide or to virus, the sequence 141 to 160-Cys has been shown to elicit helper T cell activity in the guinea-pig (Francis et al., 1987a), provided that a non-native C-terminal cysteine is present. A possible role for the cysteine may be in the formation of disulphide bonds between peptide molecules, or between peptide and proteins of host origin, thereby creating conjugates.
Conjugation of B and T cell epitopes (Cox et al., 1988) and collinear synthesis of the same (Borras-Cuesta et al., 1987, 1988) have been shown to influence the immunogenicity of peptide antigens. Furthermore, the addition of T cell epitopes to peptides with known B cell activity (Good et al., 1987; Francis et al., 1987b) has been shown to overcome major histocompatibility complex restriction, a known problem with peptide vaccines. Thus, for the development of an effective synthetic FMDV vaccine there is a clear need to demonstrate T cell epitopes within the proteins of FMDV that are recognized by a natural host.

In this paper we describe the proliferative response of cattle lymphocytes following in vitro stimulation by purified FMDV and isolated structural proteins as a preliminary to a more detailed characterization of T cell recognition of FMDV proteins and synthetic peptides.

Methods

Virus preparations

(i) Purified virus. FMDV was grown on monolayers of BHK 21 cells and purified on two sucrose density gradients as previously described (Colton et al., 1984). The strains of FMDV used were OBFS (type O, strain BFS 1860, subtype 1), ACruz (type A, strain Cruzeiro, subtype 24), SAT1 (type SAT1, strain Botswana 1/68) and ASIA (type ASIA1, strain Iran 1/73). In addition, OBFS, ACruz and swine vesicular disease virus (SVDV, strain UKG 27/72) were grown on monolayers of IB-RS2 cells and processed as for BHK-grown viruses. Purified and concentrated virus was isolated from the sucrose density gradients by flow p"ometry and the mass of recovered virus determined by the method of peak area integration (Doel et al., 1982).

(ii) Formalin-fixed virus. Fixed FMDV was prepared by treatment with 0.05% (w/v) formalin (Rowlands et al., 1972). Briefly, 1% formalin in 0.04 M-sodium phosphate buffer pH 7.6 was diluted 1:20 with virus, incubated at 20 °C for 72 h and the reaction stopped with an excess of sodium metabisulphite. The reaction mixture was immediately buffered with 8.8% (w/v) sodium bicarbonate. The fixed virus was dialysed against an excess of phosphate-buffered saline containing 1% (v/v) ox serum for 24 h, passed through a 0.22 µm Millex-GV (SLGV 025 BS) (Millipore) membrane filter and the virus content of the filtrate quantified by sucrose density gradient analysis.

(iii) Subunit antigen. Acid-denatured 12S antigen was prepared by the dilution of 1 vol. of 146S virus with 2 vol. of filter-sterilized 0.05 M-sodium dihydrogen orthophosphate pH 4.6 containing phenol red indicator. After 10 min at 20 °C the pH was adjusted to 7.6 by dropwise addition of 1 M-sodium hydroxide. The mass of 12S antigen was taken to be one-third of the starting virus, assuming that loss during acidification was minimal.

Nitrocellulose-bound FMDV polypeptides. Purified 146S FMDV was disrupted by boiling in sample buffer and the structural proteins were separated in a 12.5% (w/v) discontinuous PAGE system (Laemmli, 1970) using a Bio-Rad Mini-protein II electrophoresis tank. The resolved proteins were electrophoretically transferred to nitrocellulose according to the method of Sangar & Clark (1986). Individual proteins were identified by comparison with proteins eluted from isoelectric focusing gels (King & Newman, 1980). For proliferation assays, protein bands were visualized using Aurodye (Janssen Life Sciences Products)

and particulate antigen was prepared (Abou-Zeid et al., 1987) from strips of the nitrocellulose filter corresponding to discrete structural proteins.

Peripheral blood mononuclear cells (PBMC). Eight Friesian cattle were immunized against FMDV by inoculation of a standard cattle dose of a commercial FMDV vaccine (Coopers Animal Health) and were boosted by a second dose after 9 to 12 months. Cattle PB51, PB53, PR90, PR91 and PR92 were immunized against OBFS and PB61, PB62 and PB63 were immunized against ACruz. Animal PE58 was immunized against bluetongue virus serotype 3 (BTV-3) by inoculation of BHK 21 cell-grown attenuated virus. Blood was collected into heparin and used immediately for the preparation of PBMC. Buffy coat leukocytes were diluted with cold Eagle's minimal essential medium (MEM) and the PBMC isolated by flotation over Lymphopaque (p = 1.086) (Nycomed) for 60 min at 400 g. Cells at the interface were aspirated and contaminating erythrocytes lysed with ice-cold, 0.83% (w/v) ammonium chloride. After three washes in MEM, PBMC were diluted to a cell density of 2 × 10^6/ml in cold RPMI 1640 medium containing 2 mM-glutamine, 1 mM-sodium pyruvate, 20 mM-HEPES, 50 µM-2-mercaptoethanol and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) (complete RPMI) and kept on ice until required.

Proliferation assay. PBMC (2 × 10^6) were cultured for 5 days in 96-well, round-bottom plates in the presence of complete RPMI containing 5% heat-inactivated foetal calf serum and various concentrations of purified FMDV, SVDV or nitrocellulose-bound polypeptides. During the last 16 h of culture, 0.2 µCi [3H]thymidine ([3H]TdR) was added and the cells were harvested using an automated device (Multimash, Dynatech). Proliferation, as correlated with the amount of label incorporated into DNA, was estimated by liquid scintillation counting and expressed as a stimulation index (SI; c.p.m. test sample/c.p.m. medium alone).

Results

Lymphoblastic transformation

The optimum cell density for proliferation of PBMC stimulated with purified FMDV was found to be 2 × 10^5 to 4 × 10^5 cells/well (results not shown) and subsequent experiments were performed using 2 × 10^5 cells/well. PBMC were stimulated with a range of concentrations of the homologous virus and pulse-labelled daily with [3H]TdR for 10 days. For all animals, a peak of [3H]TdR incorporation was observed at days 5 to 6 (Fig. 1) and, although the magnitude of the response fluctuated between experiments, the general trend of the response remained constant over the period of assay. Maximum stimulation was induced using a final concentration of 1 µg/ml of purified FMDV (Fig. 2) and concentrations in excess of this were inhibitory. A second group of cattle with a similar vaccination history to that of the PB series was used to examine proliferation at times close to and distant from administration of the vaccine. The dose and temporal responses of PR90, PR91 and PR92 were found to be similar to that of the PB animals when assayed at 10 weeks post-vaccination (Fig. 3a, b). However, at 20 months post-vaccination the response of each animal...
was reduced (Fig. 3c, d) and required less antigen to stimulate a peak response (< 100 ng/ml). In general, the maximum stimulation occurred at days 3 to 4 rather than days 5 to 6, although animal PR92 displayed a biphasic response, greater at day 7. Following a further administration of vaccine the pattern and magnitude of the proliferative response closely resembled that shown in Fig. 3(a, b).

**Antigen specificity**

Although FMDV does not have a lipid envelope, cellular antigens may become associated with the virus during vaccine formulation or virus purification and thereby induce a response in vaccinated cattle. Consequently, the proliferative response was assessed in relation to both tissue culture cell antigens and purified FMDV. First, stimulation using FMDV grown in BHK or IB-RS2 cells was compared (Fig. 4), which indicated that the general characteristics of the response were similar for virus prepared in either cell type. It should be noted that neither antigen was consistently capable of stimulating a greater response than the other and that the response was variable in magnitude between assays for any given animal. No difference was observed in either the temporal or the dose kinetics of proliferation (results not shown). Secondly, the response of a control animal (PE58) was tested; this had been immunized with BTV-3 and had a demonstrable BHK-specific antibody response (results not shown), but was immunologically naive with respect to FMDV. However, no proliferation in response to purified FMDV could be detected in animal PE58. From these results it seems unlikely that the proliferative response detected was against tissue culture antigens. Specificity for FMDV was assessed by examining the response of the same animals to SVDV. At all doses and in all animals tested the response to SVDV was poor in comparison with that to FMDV (SI ≤ 3; Fig. 4) and was judged to be negative as it did not exceed the response against medium alone plus three times the standard deviation.
Fig. 3. Comparison of the in vitro proliferative response of three OBFS-vaccinated cattle (---, PR90; --, PR91; ---, PR92) at 10 weeks (a and b) and 20 months (c and d) post-revaccination. PBMC (2 × 10⁵) were cultured with OBFS, as described for Fig. 1 and Fig. 2, and proliferation was determined by the incorporation of [³H]TdR. Proliferation is expressed as a stimulation index. Panels (a) and (c) show response with time, panels (b) and (d) show response with dose.

**Response to serotypically distinct and modified virus particles**

PBMC from OBFS- and ACruz-vaccinated cattle were stimulated with homotypic and heterotypic strains of FMDV. Fig. 4 shows that cross-reactive proliferation was induced by strains of FMDV serotypically distinct from the strain used to immunize a given animal. Furthermore, with the exception of PB63, the strongest cross-reactivity identified was between OBFS and ACruz, followed by ASIA and SAT1 virus strains. Animal PB63 was unusual in that it gave a higher response with ASIA than with the homotypic virus, ACruz. This trend was consistent over a period of several months, although the magnitude of the response fluctuated from week to week.

Fig. 5 shows the proliferation obtained using 146S, 12S and fixed virus from OBFS and ACruz. Little difference was observed in the response of any given animal to the three antigen preparations. However, although the responses shown indicate a bias for the homotypic serotype, the response of each animal varied from week to week and often showed a stronger response to one or more of the heterologous virus antigens.

**Response to individual structural polypeptides**

Virus proteins were resolved by SDS–PAGE and blotted onto nitrocellulose, as described in Methods. Three proteins were resolved and their identity and purity were assessed by comparison with proteins eluted from
FMDV-specific proliferation of cattle PBMC

Fig. 5. Recognition of modified virus particles by PBMC from two OBFS FMDV-vaccinated cattle (PB51, 53; a and b), two ACruz FMDV-vaccinated cattle (PB61 and 62; c and d). PBMC (2 x 10^6) were cultured with 1 µg/ml of antigen and proliferation was determined by [³H]Tdr incorporation after 5 days. Proliferation is expressed as a stimulation index. Cells from each animal were stimulated in vitro with purified virus (146S), formalin-fixed virus (F-146S) or subunit particles (12S) prepared from OBFS (filled bar) or ACruz (hatched bar).

Fig. 6. SDS-PAGE of O1 BFS 1860 FMDV showing separation of the major structural proteins. Lane 1, Mr markers; lane 2, OBFS FMDV; lane 3, VP1; lane 4, VP2; lane 5, VP3. Lanes 3 to 5, OBFS FMDV proteins eluted from an isoelectric focusing gel.

Fig. 7. Antigen specificity of PBMC from three OBFS FMDV-vaccinated cattle (PR90, 91 and 92) and one ACruz FMDV-vaccinated bovine (PB63). PBMC (2 x 10^6) were cultured with 1:1000 of nitrocellulose-bound structural polypeptides and the proliferation was determined by [³H]Tdr incorporation after 5 days. Proliferation is expressed as a stimulation index. Cells from each animal were stimulated in vitro with VP1, open bar; VP2, hatched bar and VP3, filled bar.

isoelectric focusing gels as shown in Fig. 6 and was confirmed by Western blotting using antibodies specific for VP1, -2 and -3 (Haresnape & McCahon, 1983). The lymphoproliferative response of PB63 (36 months post-vaccination), PR90-92 (20 months post-vaccination) to each of the major structural proteins of OBFS was tested at 1:100, 1:1000 and 1:10000 dilution of antigen. Fig. 7 shows the response obtained at the optimum dose of 1:1000 and indicates that all three of the major capsid proteins were recognized by lymphocytes from the homotypically (OBFS) vaccinated cattle PR90 and PR91, whereas the homotypic animal PR92 and the heterotypically (ACruz) vaccinated animal (PB63) recognized VP3 of OBFS to a greater extent than either VP1 (PR92 and PB63) or VP2 (PB63).

Discussion

In this paper we have examined the response of bovine PBMC to 146S particles of FMDV and related antigens. The lymphoblastic response to FMDV was seen to be dose-dependent, with peak proliferation at 5 to 6 days post-stimulation. The optimum response was induced by 1 µg/ml of virus and concentrations in excess of this were inhibitory. In an earlier paper, Wardley et al. (1979) observed optimum proliferation to occur with 7.5 x 10^5 cells and 20 complement-fixing units of antigen/well (approximately 3.5 µg/ml) and to peak after 2 to 3 days stimulation. The ratio of antigen to cells in both studies is similar (approximately 5 pg/cell) and thus the more rapid response observed by Wardley et al. (1979) is probably...
due to the relative proportion of interacting cell subsets and cellular contacts, rather than the frequency of responder cells per se (Ross et al., 1987).

The duration of protection induced by vaccination is generally short lived and revaccination is usually required at 4 to 6 monthly intervals (McKercher et al., 1975), yet our results clearly identify circulating memory lymphocytes for up to 20 months post-vaccination (Fig. 3). Furthermore, the weaker but more rapid response of the 20 month compared to 10 week post-vaccination animals is indicative of maturation of the responding PBMC population. However, the variation in both the magnitude and the dose response of individual animals shows that these cells are not induced and/or maintained with equal efficiency despite the same vaccination regime. It is likely that in addition to the requirement for circulating, neutralizing antibody the persistence of circulating memory lymphocytes is a factor in determining the duration for which an animal will be protected following vaccination.

The antigen specificity of the responding cell population was assessed against a range of antigen particles. Proliferation against purified FMDV was not induced by contaminating cellular proteins as there was little difference in either the magnitude or the kinetics of the response induced by BHK or IB-RS2 cell-grown virus and also an animal with a demonstrable anti-BHK antibody response (PE58) also failed to respond to either the BHK- or IB-RS2-grown virus. The specificity for FMDV was demonstrated by the lack of response to another picornavirus, SVDV. In contrast, the response to 146S particles of different serotypes of FMDV was seen to be cross-reactive and presumably indicates recognition of conserved sequences. It should be noted that the definition of a serotype in the context of FMDV is based on both the specificity of the neutralizing antibody and the protection induced by a given virus. Even with multiple vaccination cattle remain susceptible to all FMDVs except those of the vaccine serotype or closely related virus strains.

Induction of proliferation against FMDV did not require the virus proteins to be in a native conformation, since both fixed and denatured (12S) particles stimulated similar responses to that of unmodified 146S particles. Given that the cells responding in proliferation assays are mainly T cells (Corradin et al., 1977; Lee et al., 1979) and that the recognition of antigen by T cells is independent of native protein conformation (Livingstone & Fathman, 1987; Blackman et al., 1988) the data reported here strongly support a T cell-mediated response that requires immune processing.

Proliferation assays with isolated virus proteins indicated that all three of the major capsid proteins were recognized by T cells, although in most cases the response was weak by comparison with that of animal PR91. To some extent this lends support to prediction methods for T cell sites (Margalit et al., 1987; Rothbard & Taylor, 1988), which generate a considerable number of putative sites in each of the major structural proteins (T. Collen & T. R. Doel, unpublished). Moreover, no one protein, or region of a protein, seems to have a strong predictive bias over any other. It is therefore interesting that the cross-reactive response observed in heterotypically vaccinated cattle was mainly directed at VP3 (response of PB63 shown in Fig. 7), although this is not so surprising when one considers that of the three proteins assayed VP3 has the greatest amino acid sequence conservation between serotypes.

The cross-reactivity observed between the different FMD serotype viruses should be considered in relation to their protein homology. The greatest cross-reactivity was observed between OBFS and ACruz and the least between OBFS and SAT1 (Fig. 4). This is consistent with the homology for these viruses, where OBFS and ACruz have 69.6% identity in VP1 and 80.0% identity in VP3. In contrast, OBFS has only 40.3% and 46.2% identity in VP1 and 57.8% and 63.1% identity in VP3 with SAT2 and SAT3 virus, respectively. Similarly ACruz has 40.8% and 41.7% identity in VP1 and 58.3% and 65.7% identity in VP3 with SAT2 and SAT3 virus, respectively. We did not have access to sequence data for SAT1 to make a similar analysis of homology between SAT1, OBFS and ACruz. Homology in VP2 is intermediate between those of VP1 and VP3.

In the future, studies will focus on the response of bovine T cells to synthetic peptides and enzymically generated fragments of the antibody-dominant protein VP1. However, because of the cross-reactive recognition of VP3 and the obvious merit for the inclusion of epitopes with broad stimulatory activity in a peptide vaccine, similar studies will be conducted with VP3.

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References


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