Proliferative responses of T cells primed against human rhinovirus to other rhinovirus serotypes

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Lymphocytes from mice immunized with human rhinovirus (HRV) serotypes 1A or 15 proliferated in vitro in response to HRV and the activated cells were shown to be helper T (Th) cells. Lymphocytes from mice primed with HRV-IA responded to seven of eight heterologous virus serotypes, the responses to other minor cell receptor group viruses being greater than to those belonging to the major cell receptor group. A similar bias was seen with cells from mice primed with HRV-15 in that they responded preferentially to other major receptor group viruses. This pattern of cross-serotype recognition was shown to be similar in three inbred mouse strains and was not dependent upon the major histocompatibility complex haplotype. These results have revealed that there are determinants within the viral proteins of a number of serotypes of HRV that are recognized by Th cells primed against a single HRV serotype. Thus, at the level of Th cell recognition of HRV, a cross-serotype reactivity is seen which is not reflected in the B cell antibody response to virus, which is generally highly serotype-specific.

Introduction

Human rhinoviruses (HRVs) are the major causative agents of the common cold (Stott & Killington, 1972) and possibly the most common cause of acute infections in man (Gwaltney, 1975). The virus capsid is made up of 60 protein trimers comprising three surface structural proteins, VP1, VP2 and VP3, with a fourth protein, VP4, located internally, and contains a single copy of positive-strand RNA (Mendappa et al., 1971; Rueckert, 1976). HRVs exhibit a high degree of antigenic variation, with over 100 serotypes having been identified (Hamparian et al., 1987). Although the presence of virus-neutralizing antibodies correlates with protection against disease (Doggett, 1965), anti-HRV antibodies are highly serotype-specific, demonstrating little or no cross-neutralization of other rhinovirus serotypes. The different serotypes of HRV can also be divided into two groups, major or minor, depending on the cellular receptor to which they bind (Abraham & Colonno, 1984).

HRVs cause a rapid lytic infection of the cells lining the nasal endothelium (Bynoe et al., 1961), and it has been observed that during the course of such an infection the number of circulating T cells in the peripheral blood decreases dramatically, possibly due to migration of the lymphocytes from the blood into infected tissues (Levandowski et al., 1986). These in situ lymphocytes may then exhibit cytotoxic activity and/or mediate the activity of other cells involved in the cellular immune response. An investigation of peripheral blood mononucleocytes (PBMCs) from infected human volunteers has demonstrated both non-specific and virus-specific cellular activity (Hsia et al., 1990), indicating that HRV infection activates a systemic immune response. The antigen-specific proliferation of PBMCs is indicative of the presence of virus-specific T cells, a subset of which would be T helper (Th) cells capable of stimulating antibody production by B cells. The generation of Th cells that recognize and proliferate with different HRV serotypes could be utilized in the design of synthetic human vaccines by incorporating the HRV Th cell epitopes (Francis & Clarke, 1989) with identified B cell epitopes from HRV (Francis et al., 1987; Hastings et al., 1990) or possibly other viruses.

In this study we have investigated the Th cell responses to HRV in mice of different genetic backgrounds with a view to characterizing further the T cell epitopes responsible for the cross-recognition of different serotypes of the virus.

Methods

Virus serotypes. HRV-3, -14, -15 and -49 were received from W. Barclay, MRC Common Cold Unit, Salisbury, U.K.; HRV-7 and -29
were from V. V. Hamparian, Children's Hospital, Columbus, Ohio, U.S.A.; HRV-1A was from D. C. Pevear, Sterling Winthrop, Rensselaer, N.Y., U.S.A.; HRV-1B was from The Wellcome Foundation, Beckenham, U.K.; HRV-2 was received from D. Blaas, Vienna, Austria. The viruses were plaque-purified three times and their identities confirmed using virus serotype-specific antisera.

Preparation of virus stocks. Virus was grown in OH-5 cells, a cloned line of O-HeLa cells which is highly susceptible to rhinoviruses. Stocks were prepared by infecting confluent cell monolayers with 50 µl of plaque-purified virus. After 1 h at room temperature to allow virus attachment, 20 ml of Eagle's MEM supplemented with 2% foetal calf serum (FCS) and 30 mM-magnesium chloride were added, and the flask s were incubated at 34 °C. When c.p.e. was apparent (36 to 48 h post-infection depending on virus serotype) virus was released by freezing and thawing twice, and the cell debris sedimented at 3000 r.p.m. in a bench centrifuge. The supernatant was stored in 0.5 ml aliquots at −70 °C. Large-scale virus preparations were produced by growing inoculum as above, using 0.5 ml of the stock virus to infect each flask, and then infecting roller bottle cultures containing approximately 10^6 cells with 5 ml of fresh inoculum. The bottles were stored at −20 °C when c.p.e. was complete.

Virus purification. A modification of the method described by Appleyard et al. (1990) was used. Virus was harvested from 10 to 20 roller cultures by twice freezing at −20 °C and thawing, the supernatant was clarified by centrifugation and virus was precipitated by the addition of an equal volume of saturated ammonium sulphate in 0.04 M-phosphate buffer pH 7.0. After 1 h at 4 °C, the precipitate was sedimented at 3000 r.p.m. for 40 min and resuspended in 120 ml of ice-cold Eagle's MEM containing 1% Sarkosyl and 5 mM-EDTA. Virus was pelleted by centrifugation at 33000 r.p.m. for 90 min using a Beckman T55 rotor, resuspended in 1 ml of 10 mM-Tris-Cl, 1 mM-EDTA, 0.1% 2-mercaptoethanol buffer at pH 7.5 and fractionated on a linear 15 to 45% (w/v in PBS) sucrose gradient for 2 h at 40000 r.p.m. in an SW40 rotor. Pooled 0.5 ml fractions were taken from two gradients and the virus peak was detected by measuring the A_{490} in a spectrophotometer. Fractions representing the peak were pooled, sterile filtered through a 0.22 µm filter and stored at −70 °C. The serotypes of the sucrose gradient-purified viruses were confirmed by cross-neutralization assays performed with HRV serotype-specific antisera (data not shown).

Measurement of protein concentration. The concentration of protein in the purified virus preparations was measured using a microplate assay. A bovine serum albumin standard (100 µg/ml) and the samples were serially diluted twofold in 100 µl of distilled water in eight wells of a microplate. Bio-Rad protein assay reagent (100 µl) diluted fivefold in distilled water was added and the A_{600} read using an automated plate reader. The protein concentrations were calculated using linear regression analysis of the standard curve.

Preparation of primed lymph node cells. Purified virus was diluted in sterile PBS and emulsified 1:1 with Freund's complete adjuvant (Difco) to give a final virus concentration of 2 µg/ml. Female, 8 to 10-week-old specific pathogen-free mice, either BALB/c, C3H/He or C57. Bl (obtained from Charles River), were immunized in the base of the tail with 100 µl of the experimental preparation. Seven to 10 days after immunization the draining inguinal and para-aortic lymph nodes were taken and passed through a stainless steel mesh to produce a single-cell suspension which was washed twice in ice-cold RPMI 1640 supplemented with penicillin [100 units(U)/ml], streptomycin (100 µg/ml) and 20 mM-glutamine (RPMI). Viable cells were counted and resuspended on ice at 5 × 10^6 cells/ml in RPMI supplemented with 1% normal mouse serum and 1 × 10^{-4} 2-mercaptoethanol.

Cell proliferation assay. Control antigens, concanavalin A (Con A; 500 µg/ml, Sigma) and purified protein derivative (PPD; 1000 U/ml; Evans) and purified viruses (2 µg/ml, 2 × 10^{-4} µg/ml) were diluted in RPMI and 100 µl was added in triplicate to 96-well flat-bottomed tissue culture plates; controls containing medium alone were also included. Cell suspension (100 µl) was then added and incubated at 37 °C in a 5% CO_2 humidified atmosphere for 4 days. The cultures were then pulse-labelled with [1 µCi] {^3}H]thymidine (Amersham) for 16 h prior to harvesting onto filter mats using an automated cell harvester (Skatron), and measuring the amount of thymidine incorporated into the cells by liquid scintillation counting (LKB Betaplate). The results were expressed as the mean of three determinations minus the background reading obtained using cells incubated with medium alone.

Antibody and complement depletion assay. Tissue culture supernatants containing monoclonal antibodies (MAbs) against CD4 (GK1.5), CD8 (3.168) and Thy1 (H.0139), from B. Chain, University College, London, U.K., were used in conjunction with guinea-pig complement (Seralab) to deplete different populations of primed lymph node cells. Cells (1 ml, 2 × 10^5/ml) were incubated with 1 ml of MAb at 4 °C for 45 min, washed twice with ice-cold RPMI, and then incubated at 37 °C with complement at a final dilution of 1:20 for 30 min. Cells not treated with MAb were also included as a control. After washing twice, viable cells were counted, and the preparations were resuspended at 5 × 10^6 cells/ml and assayed in triplicate for proliferation with control or specific antigens, as described above. Cells were also cultured with antigens in the presence of TIB 120, a major histocompatibility complex (MHC)-binding agent supplied by B. Chain, added to a final concentration of 20 µg/ml.

Results

Lymphocytes from BALB/c mice immunized with HRV-1A proliferate in response to HRV-1A and heterologous HRV serotypes

Initial experiments with BALB/c mice indicated that a proliferative response to HRV-1A was obtained after immunization with 0.2 µg of purified virus and that the peak response occurred 7 to 10 days post-immunization, at in vitro antigen concentrations of 2 × 10^{-1} to 2 × 10^{-3} µg/ml (data not shown). The proliferative response of HRV-1A-primed cells to eight other HRV serotypes was investigated over a range of antigen concentrations from 2 to 2 × 10^{-3} µg/ml. Cell proliferation was observed with homologous virus and with seven other HRV serotypes; only one, HRV-15, gave little or no proliferation (Fig. 1). The responses to the different serotypes were varied; HRV-2 and -29 stimulated cells as well as HRV-1A, with high levels of [^3]H]thymidine incorporation at low concentrations of virus. HRV-1B and -49 also gave good incorporation, but at higher virus concentrations, whereas HRV-3, -7 and -14 stimulated proliferation to a lesser extent and only at higher antigen concentrations.

Proliferative responses to HRV-1A and other virus serotypes are observed in different mouse haplotypes

To determine whether the response to heterologous HRV serotypes and the pattern of cross-reactivity seen with BALB/c (H-2^d) mice was unique to that haplotype,
T cell responses to rhinoviruses

Fig. 1. Proliferation of HRV-1A-primed BALB/c (H-2d) lymph node cells in response to nine serotypes of HRV (HRV-1A, -1B, -2, -3, -14, -15, -29 and -49; a to i). [3H]Thymidine incorporation was determined in triplicate for each concentration of virus tested, the mean taken and the background c.p.m., obtained from cells incubated with medium alone, was subtracted. The results represent the mean of three experiments and error bars represent the S.E.M.

Fig. 2. Proliferation of HRV-1A-primed C57.BL (H-2b) cells with different HRV serotypes (a to i, see legend to Fig. 1). Results represent the mean of two experiments and error bars the S.E.M.

Fig. 3. Proliferation of HRV-1A-primed C3H.He (H-2k) mouse lymphocytes with HRV serotypes (a to i, see legend to Fig. 1). The results represent the mean of two experiments and error bars the S.E.M.

C57.BL (H-2b) and C3H.He (H-2k) mice were immunized with HRV-1A and assayed for lymphocyte proliferation with the same panel of viruses. H-2b mice (Fig. 2) responded well to homologous virus and the heterologous serotype, HRV-29, as did H-2k mice (Fig. 3). Lymphocytes of both haplotypes also proliferated with HRV-1B, -2 and -49, but gave lower levels of [3H]thymidine incorporation at higher antigen concentrations than in the response to HRV-1A. The responses to HRV-3 and -14 were lower still (Fig. 2 and 3). Lymphocytes from neither mouse strain gave a proliferative response with HRV-7 or HRV-15.

HRV-15-primed lymph node cells exhibited different proliferative responses to those observed with HRV-1A-primed cells

Since little or no proliferative response to the major receptor group virus HRV-15 was demonstrated using lymphocytes from minor receptor group HRV-1A-primed animals, BALB/c mice were immunized with HRV-15, and tested for their response to the HRV panel (Fig. 4). The level of the response to the homologous HRV-15 was much lower than that of HRV-1A-immunized mice to HRV-1A, and occurred only at the two highest virus concentrations. Cells proliferated in response to HRV-3 to a similar degree, but responses to the other major receptor group viruses, HRV-7 and -14, were observed only at the highest antigen concentration, 2 μg/ml. Little or no response was seen to the minor group viruses, HRV-1A, -1B, -2, -29 and -49.

Proliferation in vitro is due to HRV and not non-specific antigens

Lymph node cells from unimmunized mice were tested for non-specific proliferation with each of the purified viruses. There was no proliferation in response to the three highest antigen concentrations used in the previous
assays, with the exception that HRV-2 gave low [\(^3\)H]thymidine incorporation at the highest antigen concentration, 2 µg/ml. To demonstrate further that proliferation was antigen-specific and not due to other components in the virus preparations, HRV-1A-primed cells were cultured with a range of dilutions of sucrose and HeLa cell extract, containing FCS and cell-associated antigens, and no proliferation was observed (data not shown). In all proliferation experiments the cell controls in medium alone gave low backgrounds of <1000 c.p.m., but responded well to the positive control stimulants Con A and PPD (data not shown).

The proliferating lymph node cells are CD4\(^+\) Th cells

The phenotype of the proliferating cells was determined by pretreating HRV-1A-primed lymph node cells from BALB/c mice with MAbs to cellular antigens and complement to deplete different populations of cells. The viable cell count obtained after treatment indicated that depletion had occurred (data not shown). Incorporation of [\(^3\)H]thymidine was eliminated after treatment with the anti-Thy1 MAb (H.0139), indicating that the cells proliferating in response to HRV-1A were T cells (Table 1). After treatment with the anti-CD4 MAb (GK1.5), [\(^3\)H]thymidine incorporation was reduced by approxima-

<table>
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<th>HRV-1A (µg/ml)</th>
<th>None</th>
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<th>CD8</th>
<th>Thyl</th>
<th>TIB 120</th>
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* [\(^3\)H]Thymidine incorporation (c.p.m.).
† ND, Not determined.

tely 90\% of that observed with control cells treated with complement alone, whereas the anti-CD8 MAb (3.168) had no effect. Moreover, cells cultured with HRV-1A in the presence of TIB 120 did not proliferate after virus challenge, demonstrating their I-A/I-E restriction. All cells responded to Con A and PPD, with the PPD response affected by antibody treatment in a manner similar to that observed with virus (data not shown). Background levels of proliferation of cells in medium alone were <400 c.p.m.

**Discussion**

In this study we have investigated the ability of different serotypes of HRV to cause proliferation of murine lymph node cells primed with specific serotypes, one from the minor receptor group and the other from the major receptor group.

Cellular proliferation, measured by incorporation of radiolabelled thymidine into the DNA of the dividing cells, was shown to be antigen-specific and the proliferating cells were identified as Th cells, being Thy1\(^+\), CD4\(^+\) and CD8\(^-\) and restricted in their recognition of antigen by MHC class II I-A/I-E molecules.

The observation that a single inoculation of 0.2 µg of purified HRV-1A emulsified with Freund’s complete adjuvant was capable of priming Th cells specific for virus indicated that the virus was a potent immunogen. Moreover, optimal proliferation occurred *in vitro* with 2 to 20 ng of virus, amounts which are 10- to 100-fold lower than those reported for another picornavirus, poliovirus (Wang *et al.*, 1989), for mice which had been primed with 2 µg of virus. Higher concentrations of HRV-1A appeared to have an inhibitory effect on cell proliferation *in vitro*.

HRVs are highly immunogenic in experimental animals, with low doses of virus raising high titres of antibodies directed against the surface epitopes of the virus in mice (M. J. Francis & G. Z. Hastings, unpublished data). Neutralizing antibodies to most virus serotypes have been shown to be highly serotype-specific (Cooney *et al.*, 1982), with only a small number of the 90
serotypes tested exhibiting any cross-reactivity with heterologous viruses. Similarly, in humans immunity to infection by a specific serotype of HRV relies upon the presence of pre-existing antibody to that serotype (Doggett, 1965), with antibodies to other serotypes giving little or no cross-protection. In humans infected experimentally with an unspecified serotype of HRV, lymphoblastic cells given prior to infection have been shown to proliferate to the virus when no virus-neutralizing antibodies had been detected, implying that cells previously primed by infection with different virus serotype(s) recognize determinants present on the challenge virus (Hsia et al., 1990).

Although there is an element of serotype-specific responsiveness to HRV-1A similar to that described for poliovirus by Leclerc et al. (1991), this study has shown that cross-serotype recognition of HRVs does occur at the level of Th cells, in agreement with the findings of Wang et al. (1989) and Karrak et al. (1991) which demonstrated cross-serotype Th cell responses to poliovirus in mice. Cross-serotype Th cell responses have also been seen with foot-and-mouth disease virus in lymphoblastic bovine cells (Collen & Doel, 1990).

HRVs can be divided into two groups depending on the cellular receptor to which they bind (Abraham & Colonna, 1984). Most serotypes bind to the major receptor (Uncapher et al., 1991), now identified as the ICAM-I molecule (Greve et al., 1989; Staunton et al., 1989), with a lesser number binding to the uncharacterized minor receptor (Mishak et al., 1988). These two groups of viruses gave different proliferation profiles with HRV-1A-primed cells, which proliferated preferentially with other minor receptor group viruses, whereas major receptor group-primed cells respond almost exclusively to other major receptor group HRV serotypes.

These results indicate that there may be some difference between the abilities of the two receptor groups of virus to exploit the Th cell response. Major group viruses bind only to higher primate cells (Colonna et al., 1986), whereas minor group viruses have also been shown to bind to mouse L cells (Yin & Lomax, 1983). In the assays described in this paper it is possible that HRV-1A becomes bound to mouse cells in vivo and/or in vitro, and that the uptake of the antigen into the cells was thus facilitated. This binding could then affect the processing of the virus and its subsequent presentation by the cells. This may explain the finding that low doses of HRV-1A prime and induce proliferation of murine Th cells, but that higher concentrations of major group virus are required in vitro, and probably in vivo, to produce similar levels of proliferation to those obtained with minor group viruses. Receptors for both groups of virus are expressed on human cells, and a similar mechanism of virus uptake could occur in individuals infected with HRV.

This difference in proliferative response to the two receptor groups of virus was also observed in two other mouse strains with different genetic backgrounds, demonstrating that the T cell recognition of HRV is not restricted by MHC haplotype. Differences in the T cell proliferative responses of inbred mouse strains have been reported with poliovirus (Wang et al., 1989) and Theiler's mouse encephalitis virus (Clatch et al., 1987). In contrast, comparison of the stimulation indices for the three mouse strains used in this study showed that they gave similar proliferative responses to most of the virus serotypes.

In a previous study with HRV-2 we used a predictive algorithm (Rothbard, 1986) to identify five potential T cell epitopes from the amino acid sequence of the virus structural proteins (Skern et al., 1985). These were synthesized in conjunction with an identified HRV-2 VP2 B cell epitope (Francis et al., 1987), which was unable to elicit an antibody response in the absence of Th cell determinants, and used to elicit antibodies in mice. One was found not to be restricted by MHC haplotype in both outbred mice and six inbred mouse strains (Francis et al., 1989), implying that universally recognized T cell epitopes may exist on HRV. It is of interest that this particular epitope (VP1 amino acids 251 to 260) is not highly conserved between the HRVs for which amino acid sequences are available (Palmenberg, 1989). HRV-1A has been used in this study due to the availability of structural coordinates (Kim et al., 1989), which enables the relationships of different epitopes, whether T or B cell, present in the virus structural proteins to be examined at the molecular level.

Having demonstrated that cross-reactive Th cells to HRVs can be generated in mice of different genetic backgrounds, further studies will attempt to identify the specific regions of the structural proteins of HRV-1A which are involved in T cell recognition of virus.

References


