Identification of Mengo virus T helper cell epitopes

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To identify Mengo virus-specific T cell epitopes in mice (the natural host for the virus), lymph node cells were obtained from BALB/c (H-2a) mice, previously immunized with u.v.-inactivated virus, and stimulated in vitro with each of 116 overlapping peptides (10 to 18 residues long) covering the entire capsid coding region (834 amino acids). T cell epitopes were defined on the basis of specific peptide-induced lymphocyte proliferation. Where proliferation occurred, immunological characterization showed that it was the CD4+ T helper (Th) cell subpopulation that was responsible for the Mengo virus-specific response. Surprisingly, no Mengo virus Tn cell epitopes were found in capsid protein VP1 or VP4. Six peptides in VP2 (residues 1 to 15, 99 to 108, 118 to 132, 133 to 147, 227 to 236 and 247 to 256) identified the positions of separate Th cell epitopes, and two overlapping peptides (residues 173 to 182 and 178 to 192) defined an additional Th cell immunogenic sequence. Three individual peptides in VP3 (residues 46 to 58, 136 to 150 and 198 to 212) and two overlapping peptides (residues 1 to 15 and 11 to 20) also represent Th cell epitopes. Similar assays with C57BL/6 (H-2b) and SJL/J (H-2s) mice showed that the pattern of recognition of these peptides was H-2 restricted. Each of the previously identified sites of B cell antigenicity in VP2 and VP3 are associated with one Th epitope. Comparison of the experimentally determined Th epitopes with potential T cell epitopes identified by several predictive strategies revealed only a low correlation between authentic and predicted epitopes.

Introduction

Mengo virus is a member of the genus Cardiovirus of the family Picornaviridae and is a natural pathogen of mice. All picornaviruses contain a positive-strand RNA genome coding for a single polyprotein; subsequent to translation this is proteolytically processed into a number of viral proteins, including the four structural proteins VP1, VP2, VP3 and VP4. Sixty copies of each of the structural proteins form the icosahedral capsid of the virus, the structure of which has been determined to a resolution of 0.30 nm (Luo et al., 1987; Krishnaswamy & Rossmann, 1990). This makes it possible to correlate immunologically reactive entities (epitopes) with specific structural elements in the viral capsid. Sequencing of naturally occurring mutants of the virus that escape neutralization by murine monoclonal antibodies (MAbs) has identified B cell epitopes in VP2 and VP3 regions that are located on the exterior surface of the capsid in the vicinity of a putative receptor-binding depression called the ‘pit’. These residues form a single composite neutralization site on the Mengo virion (Boege et al., 1991).

In nature, mice appear to be the reservoir for Mengo virus and the closely related encephalomyocarditis (EMC) virus. These agents have been transmitted to a variety of species and have caused serious epizootics in pigs (during the period 1986 to 1991, mouse-borne cardiovirus outbreaks were reported in piggeries in southeastern Australia, Greece, Minnesota, Western Australia and Quebec). Porcine cardiovirus disease is associated with reproductive failure or abortions in sows and systemic infection of newborn or young animals occurs with a resultant mortality of 15 to 20% (reviewed by Scraba, 1994). Laboratory mice inoculated with Mengo virus (intraperitoneal LD50 > 105 p.f.u.) develop a central nervous system (CNS) disease which resembles human CNS disease caused by enteroviruses (Campbell & Colter, 1965; Stringfellow et al., 1974). The virus replicates actively in the spinal cord and brain, and also in the lymph nodes (Ln), spleen, heart, lungs, pancreas and kidneys. Infected animals die of a severe, diffuse meningoencephalitis (Veckenstedt, 1974; Guthke et al.,

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Mengo virus infection of mice has been used as a model for the meningoencephalitis caused by human enteroviruses and a test system for the prophylactic efficacy of antiviral compounds (Veckenstedt & Horn, 1974).

The interaction of antibody and viral antigen has been examined in detail for a number of viruses, but this represents only one component of the adaptive immune response. The immune reaction to viral antigens, including the development of an efficient antibody response, is primarily T cell dependent. One example of this is the induction of proliferation of the major histocompatibility complex (MHC) class II-restricted CD4+ T helper (T_h) cells in response to antigen. These cells secrete cytokines which, in turn, mediate a variety of immune responses including the activation of antigen-specific B cells (Rheinherz & Schlossman, 1981). In addition, the subpopulation of class II-restricted T helper cells (T_h,1) has also been implicated in direct cell-mediated immunity including the lysis of virally infected target cells (Coffman et al., 1988; Mosmann & Coffman, 1989; Pallidino et al., 1991).

The present study was undertaken to examine the murine T_h cell response to immunization with u.v.-inactivated Mengo virus, to map the T_h-stimulating peptides with respect to their locations in the viral capsid and to determine their spatial relationships with known B cell epitopes. Overlapping peptides covering the entire amino acid sequences of VP1, VP2, VP3 and VP4 were systematically evaluated for their ability to induce a proliferative response in vitro in LN cells from BALB/c mice that had been primed with inactivated virus. Peptides capable of inducing a significant proliferative response were located in proteins VP2 and VP3. Two of these, one in each protein, were found to overlap with sequences contributing to the Mengo virus composite B cell-antigenic determinant. We also investigated the H-2 restriction of these epitopes in C57BL/6 and SJL/J mice. The position of the identified epitopes was examined in relation to the position of other picornavirus T_h cell epitopes in mice and in relation to potential T cell epitopes identified by predictive algorithms based upon specific structural and sequence motifs.

**Methods**

**Mice.** Relevant regulations regarding the use of animals (Canadian Council on Animal Care) were followed during the course of this research. Female BALB/c (H-2b), C57BL/6 (H-2b) and SJL/J (H-2a) mice 6 to 8 weeks of age were bred in either the Research Institute of Scripps Clinic breeding colony, the Heritage Rodent Facility of the University of Alberta or obtained from commercial sources (Charles River (Canada) or Jackson Laboratories).

**Virus purification and inactivation.** The M plaque variant of Mengo virus (Ellem & Colter, 1961) was produced in mouse L929 cells in roller bottle cultures and was purified as described previously (Boege et al., 1986; Ziola & Scraba, 1974). Purified virus (550 μg/ml), for both the immunization of mice and in vitro proliferation assays, was inactivated by u.v. irradiation (253.7 nm, 200 μW/cm², 10 cm from source). The minimum irradiation time required for complete inactivation was determined by standard plaque reduction assay (Ellem & Colter, 1960) of irradiated samples.

**Immunization.** Mice were injected subcutaneously at the base of the tail or in a foot pad with 50 μl of complete Freund's adjuvant (CFA) (Difco) emulsion into which either 5 μg of u.v.-inactivated Mengo virus (found to be optimal in initial experiments), 1.5 μg of isolated VP1, VP2 or VP3 capsid proteins, or 100 μg grade V ovalbumin (Sigma) was incorporated. The dose of individual capsid protein for immunization was chosen to represent approximately the same dose of each individual polypeptide as is given in a 5 μg injection of whole virus. For each set of ten peptides and controls, three mice were immunized and the lymph node cells were pooled from all three. All assays were done in triplicate. The response to immunization was monitored by ELISA and neutralization assays of serum collected from mice prior to sacrifice.

**Detection of anti-Mengo virus antibodies.** Serum samples were analysed for the presence of anti-Mengo virus antibodies by ELISA. Microtitre plates (Fisher Scientific) were coated overnight with 200 ng/well of purified Mengo virus in ELISA coating buffer (13.5 mM-Na₂CO₃, 35 mM-NaHCO₃ pH 9.6). Plates were blocked for 1 h at room temperature with 1% BSA (Sigma) in PBS prior to the addition of test sera. Sera, serially diluted from 1:100 to 1:6400 in PBS containing 0.05% Tween 20 (Sigma) (PBS/Tween), were then added to the plates. After a 2 h incubation at room temperature they were washed with PBS/Tween. Affinity-purified goat anti-mouse IgM+IgG+IgA alkaline phosphatase conjugate (Cedarlane) diluted 1:1500 in PBS/Tween was added and the plates were incubated as before. After washing, the enzyme substrate disodium p-nitrophenyl phosphate (Sigma) (10 mg dissolved in 10 ml of 10% diethanolamine) was added. Following a 10 min incubation period the absorbance at 410 nm was determined.

**Serum neutralizing assay.** Mice L929 cells were continuously cultured in 1 litre spinner flasks in modified basal medium Eagle (BME; Flow Laboratories) to which was added 10% fetal bovine serum (FBS; Flow Laboratories) and 1% solutions of penicillin/streptomycin (200 units/ml penicillin, 100 μg/ml streptomycin). Cells were plated at 5 x 10⁶ cells/well in 96-well tissue culture plates (Corning), the wells were washed with PBS/Tween. Affinity-purified goat anti-mouse IgM+IgG+IgA alkaline phosphatase conjugate (Cedarlane) diluted 1:1500 in PBS/Tween was added and the plates were incubated as before. After washing, the enzyme substrate disodium p-nitrophenyl phosphate (Sigma) (10 mg dissolved in 10 ml of 10% diethanolamine) was added. Following a 10 min incubation period the absorbance at 410 nm was determined.

**Peptide synthesis.** Ten to 18-mer peptides, overlapping by five residues at the amino and carboxy ends, were synthesized based upon the amino acid sequence of Mengo virus capsid polypeptides (Boege et al., 1991). A multiple simultaneous peptide synthesis method in polypropylene mesh 'tea bags' (Houghten, 1985) or symmetrical anhydride chemistry (Hagenmaiser & Frank, 1972; Merrifield, 1963) on an Applied Biosystems 430A or a Beckman Model 990 automated peptide synthesizer employing t-butyloxycarbonyl N~ and benzyl type side-chain protection was used for peptide synthesis. Peptides were cleaved from the resin and side-chain protecting groups were removed with hydrofluoric acid. The purity of all peptides (70 to 90%) was determined by reverse-phase HPLC and amino acid composition was verified by amino acid analysis. The majority of cleaved 'tea bag' peptides were used without further purification, whereas those from commercial sources (Alberta Peptide Institute; Chiron Mimmotopes) were partially purified by ion-exchange or reversed-phase HPLC and analysed by mass spectrometry.
Isolation and purification of individual capsid proteins VP1, VP2 and VP3. Individual capsid proteins were separated by SDS-PAGE in 12% acrylamide gels. Protein was eluted from excised bands into 150 mM-N-methyl morpholine (pH 8.0) and SDS was removed by extensive dialysis against PBS. Purity of individual polypeptides was assessed on polyacrylamide gels and the protein concentrations was estimated by the examination of staining intensities relative to known concentrations of capsid protein.

In vitro T cell proliferation assay. Antigen-specific T cell proliferation assays were performed using popliteal, superficial and deep inguinal and periaortic immune draining LN. LN were aseptically removed and a single cell suspension of LN cells was prepared, washed twice in RPMI 1640 medium containing 20 mM-L-glutamine and penicillin/streptomycin (200 units/ml penicillin, 100 µg/ml streptomycin). The cells were resuspended at a concentration of 5 x 10^6 cells/ml in the same medium supplemented with 2% normal murine sera or FBS that had been screened for the absence of non-specific proliferation activity. The cells were cultured in 0.2 ml of medium at a final concentration of 5 x 10^6 cells/well in 96-well flat-bottomed tissue culture plates (Corning) in the presence of antigens. In all assays the non-specific mitogen concanavalin A (conA) at the experimentally determined optimal concentration of 10 µg/ml was used as a positive control for lymphocyte proliferation. Cellular proliferation was measured by the incorporation of [3H]thymidine (1 µCi/well; 20 Ci/mmol) (New England Nuclear) into DNA during the last 16 h of a 72 h culture at 37 °C and 5% CO_2. All cultures were set up in triplicate, harvested onto glass fibre filter paper using a cell harvester (Skatron) and the radioactivity was counted in a Beckman LS scintillation counter. Data are presented either as mean c.p.m. or as stimulation index (S.I.). The S.I. is defined as the mean c.p.m. obtained for a given antigen divided by the mean c.p.m. for media controls. Stimulation indices greater than three were treated as significant based on values obtained for non-Mengo virus control peptides.

Immunological characterization of T lymphocyte population. The L3T4 (CD4) rat IgG2b anti-mouse monoclonal antibody (MAb) (Cedarlane) and the Ly2 (CD8) rat IgG2b anti-mouse MAb (generously supplied by Dr. V. Paetkau, Department of Biochemistry) were utilized for in vitro depletion assays. LN cells (5 x 10^6 cells/ml; 100 µl/well), isolated from mice immunized with u.v.-inactivated Mengo virus, were plated in the presence of 1:0 and 0:1 µg/ml homologous virus and 70 to 1700 ng/well of the L3T4 or the Ly2 MAb. Cells not treated with either MAb were used as a control. The plates were incubated for 72 h at 37 °C in 5% CO_2. The cells were pulsed with [3H]thymidine (10 µCi/well) for 16 h, harvested and cell-associated radioactivity was measured as in the in vitro proliferation assay.

Computer analysis of Mengo virus capsid protein sequences to predict T cell sites. The computer program, TSites (Feller & de la Cruz, 1991) was used to examine the amino acid sequences of the Mengo virus capsid proteins for predicted T cell sites. It is based on four different epitope-predicting algorithms. Firstly, correlation between alphahelical periodicity and amphipathicity and the antigenicity of many T cell sites (De Lisi & Berzofsky, 1985) was examined. The AMPHI algorithm (Margalit et al., 1987) is used with a scanning window of 11 residues. Secondly, a search is conducted for sequence motifs identified by Rothbard & Taylor (1988) in sequences recognized by MHC class I and class II molecules. These motifs consist of a four amino acid pattern of charged/glycine-hydrophobic-hydrophobic-polar/glycine residues and a five amino acid pattern of charged/glycine-hydrophobic-hydrophobic-proline-polar/glycine residues. The final two prediction strategies search for sequences with analogy to ovalbumin peptides which are known to bind to the murine MHC class II (Ia) molecules (Seite et al., 1989). The third algorithm searches for peptides predicted to bind to the I-A^d molecule (the 'D' motif) and the fourth algorithm identifies peptides binding to the I-E^d molecule (the 'd' motif).

Results

Antibody responses of Mengo virus immunized BALB/c mice

Sera from mice immunized with inactivated Mengo virus were examined for IgM, IgA and IgG content and for virus neutralization activity. Combined IgA, IgG and IgM levels in the sera of individual mice were measured by standard ELISA using anti-mouse combined IgA, IgG and IgM alkaline phosphatase-conjugated anti-
bodies. Sera removed from all three strains of mice the day of the assay consistently contained levels of circulating anti-Mengo virus antibodies detectable in ELISA tests at 1:5000 dilution.

To examine whether immunization with inactivated Mengo virus could induce the production of neutralizing antibodies the sera from individual mice were tested in a micro-neutralization assay. Sera from all three strains of mice consistently neutralized Mengo virus at 1:400 to 1:800 dilutions (data not shown).

**T cell proliferative responses to Mengo virus**

LN cells, obtained from BALB/c mice injected with 5 µg of u.v.-inactivated Mengo virus or 100 µg ovalbumin, were stimulated *in vitro* at 7, 9, 10 and 14 days post-immunization with homologous virus or ovalbumin. The optimal response to Mengo virus was seen at 7 days post-immunization. The specificity of the T cell response to antigen was demonstrated by *in vitro* stimulation of LN cells with inactivated Mengo virus and ovalbumin. LN cells from ovalbumin-immunized mice proliferated in response to the presence of ovalbumin, whereas their response to Mengo virus was essentially negligible at all antigen concentrations (Fig. 1a). Similarly, Mengo virus induced the proliferation of LN cells from Mengo virus-primed mice whereas ovalbumin failed to stimulate them (Fig. 1b). These results demonstrate that the T cell responses *in vitro* is specific for the antigen to which the cells have been exposed *in vivo*. In all of the assays the highest level of proliferation in Mengo virus-primed LN cells was seen with 0.1 µg/ml inactivated virus. Although the responses of individual mice varied considerably, the response to Mengo virus was generally about 50% of the proliferative response of lymphocytes stimulated with conA under the same conditions (data not shown).

**Immunological characterization of lymphocytes specific for inactivated Mengo virus**

Lymphocytes from Balb/c mice inoculated with u.v.-inactivated Mengo virus were incubated with the optimal concentration of homologous virus and with varying concentrations of anti-CD4 or anti-CD8 MAbs to determine which subpopulation of lymphocytes is responsible in the *in vitro* proliferation assays. Addition of the anti-CD8 MAb did not reduce the *in vitro* response to inactivated virus compared to the control, whereas anti-CD4 antibody was able to reduce the response to less than 20% of control values at the highest concentration of antibody used (Fig. 2). These results indicate that it is the CD4+ helper T cell population of lymphocytes that is responsible for the proliferative response of the mixed lymphocyte population from LN-derived cultures. It is likely that the increase in proliferative response observed with increasing concentrations of anti-CD8 MAb is due to the suppression of the activity of the subpopulation of CD4+CD8+...
regulatory suppressor T lymphocytes (discussed in Baker, 1993).

**Antigenicity of individual capsid proteins VP1, VP2 and VP3**

Lymphocytes were isolated from BALB/c mice that had been immunized with purified Mengo virus capsid proteins VP1, VP2 or VP3 (it was not possible to isolate sufficient quantities of VP4 to immunize mice). Cultured LN cells were stimulated *in vitro* with u.v.-inactivated Mengo virus to determine whether each protein contains T cell epitopes recognized in BALB/c mice. Lymphocytes from both VP2- and VP3-injected mice proliferated in the presence of inactivated Mengo virus, but VP1-primed lymphocytes were only weakly stimulated (Fig. 3). It was not possible to isolate sufficient quantities of the individual capsid proteins to use as antigen in the *in vitro*
The positions of the T cell-stimulating peptides in the three-dimensional structures of VP2 and VP3 are shown in Fig. 5. Previously identified B cell sites (Boege et al., 1991) are also indicated on these ribbon diagrams. Peptide K107 covers residues 133 to 147 of VP2 and overlaps the region of B cell antigenicity at residues 144, 145 and 147. The B cell site in VP3 defined by residues 57, 61 and 68 is partially overlapped by the T cell epitope containing stimulatory peptide K130 (residues 46 to 58).

H-2 restriction of the proliferative response to Mengo virus T helper cell epitopes in BALB/c mice

To examine the H-2 restriction of the peptide-stimulated proliferative responses found in BALB/c mice, parallel in vitro proliferation assays were done with LN cells from C57BL/6 (H-2b) and SJL/J (H-2s) mice that had been immunized with u.v.-inactivated Mengo virus. In all three mouse strains the VP2 peptides K89, K102 and K113 as well as the VP3 peptides K125 and G135 were recognized as T cell epitopes. With the other peptides the pattern of recognition of T cell epitopes varied among the different strains. In C57BL/6 mice, peptides K105, K107, K120, K123 (VP2), K124 and K130 (VP3) also induced significant stimulation of cell growth. LN cells from immunized SJL/J mice responded only to the five commonly recognized peptides (K89, K102, K113, K125 and G135); none of the other peptides were capable of inducing significant proliferative responses.

Analysis of predictive strategies for identifying T cell epitopes

The amino acid sequences of all four Mengo virus capsid proteins were analysed with the computer program TSites (Feller & de la Cruz, 1991) for the prediction of T cell epitopes. Fig. 7 indicates the sequences predicted by each of the four searching strategies to be possible T cell epitopes for capsid proteins VP2 and VP3. As well, the positions of the peptides containing the experimentally identified T cell sites in VP2 and VP3 are shown. Similarly to VP2 and VP3, VP1 and VP4 have a number of regions predicted to be T cell epitopes containing stimulatory peptide K107 (residues 133 to 147). The B cell site in VP2 defined by residues 57, 61 and 68 is partially overlapped by the T cell epitope containing stimulatory peptide K130 (residues 46 to 58).

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Mengo virus T helper cell epitopes

sites. Interestingly, peptides K107 and K112 are not predicted by the TSites program to be antigenic although each does stimulate a high level of lymphocyte proliferation in the in vitro assays. The VP3 peptides K125, K130 and K151 are predicted by one or more search strategy to be antigenic but VP3 also contains sites within peptides K124 and G135 which are not predicted by the TSites program.

Discussion

We have shown that immunization of mice with inactivated Mengo virus induces a CD4+ T cell response. Induction of this response is generally believed to occur through the uptake of exogenous non-replicating and soluble antigens into the endosomal pathway by antigen-presenting cells. The antigen is processed into peptide
fragments which are presented on the cell surface in association with MHC class II molecules. Interaction of the peptide bound to the MHC class II molecule with the T cell receptor of CD4\(^+\) T helper cells stimulates their proliferation. Although induction of a CD8\(^+\) T cell-specific response to soluble antigen through peptide presentation by MHC class I molecules has been demonstrated in a number of systems (Raychaudhuri & Morrow, 1993), we observed no CD8\(^+\) lymphocyte response to u.v.-inactivated Mengo virus.

Thirteen peptides that contain T\(_h\) cell epitopes were identified in Mengo virus capsid proteins VP2 and VP3; no epitopes were identified in VP1 or VP4. At the time the peptide panel was synthesized for this study peptides 10 to 18 residues in length were chosen. Based on more recent observations that MHC class II bound peptides tend to be 12 to 24 residues in length it is possible, depending on the positions in the peptides of residues that are critical for binding, that T cell epitopes could have gone undetected using peptides as short as 10 residues (Brown et al., 1993). The immunodominance of VP2 and VP3 was confirmed by the \textit{in vitro} stimulation of LN cells from BALB/c mice immunized with purified capsid proteins VP1, VP2 or VP3: \textit{in vitro} stimulation with inactivated Mengo virus induced high levels of lymphocyte proliferation in VP2- or VP3-primed LN cells, whereas the proliferation of VP1-primed LN cells was minimal. The low level of stimulation seen with the VP1-primed LN cells does suggest the presence of weak T cell sites in VP1, but these were not detectable in the \textit{in vitro} proliferation assay with peptides. It is likely that T\(_h\) cell epitopes in VP1 and VP4 (as well as additional ones in VP2 and VP3) do exist but are not recognized by the H-2\(^d\) MHC molecule which binds the 13 different peptides in VP2 and VP3.

Fig. 5 shows the positions of the known B cell epitopes of Mengo virus together with the T cell epitopes identified in this study on ribbon diagrams of VP2 and VP3. The two linear regions of B cell antigenicity are each partially overlapped by a T cell epitope. Linkage of B and T cell determinants has been observed with several other viruses and suggests that the existence of B and T cell epitopes in close proximity is a factor in determining the efficiency of an antibody response to that site (Kutubuddin \textit{et al.}, 1992; LeClerc \textit{et al.}, 1991, 1993). For both VP2 and VP3 the T\(_h\) cell epitope is linked to the amino end of the associated B cell epitope and there is evidence that the orientation of the T cell epitope with respect to the B cell epitope is important for promoting production of high-affinity antibodies. For example, it has been demonstrated that immunization of mice with chimeric peptides composed of a T cell epitope linked to the amino terminus of a B cell epitope induces the production of higher-affinity antibodies than does immunization with peptide containing the epitopes in the opposite orientation (Partidos \textit{et al.}, 1992). This ob-
observation is potentially relevant for the design of synthetic vaccines, and it is possible that the orientation of epitopes may play a role in the selection of immunodominant sites in intact pathogens.

Comprehensive studies of picornavirus T cell epitopes have been reported for rhinovirus and poliovirus (Hastings et al., 1993; Mahon et al., 1992). Overlapping synthetic peptides covering the entire capsid protein sequence for human rhinovirus type 1A were used to identify TH cell epitopes in BALB/c (H-2^d), CBA (H-2^s) and C57BL/10 (H-2^b) mice. For the BALB/c mice, only four TH epitopes (peptide S.I. > 2) were found: one in VP1 (residues 211 to 230), one in VP2 (101 to 120) and two in VP3 (161 to 180 and 181 to 200). The VP2 peptide 101 to 120 was also active in CBA mice, but not in C57BL/10 mice; the other three rhinovirus TH epitopes were H-2 restricted. All of these epitopes are found in elements of the eight-stranded beta-barrel which composes the interior core of each of the capsid polypeptides. None of the rhinovirus epitopes recognized by BALB/c mice correspond structurally with the Mengo virus epitopes that we have identified. Among the T cell epitopes found for poliovirus in mice (Mahon et al., 1992) is one in the loop between the beta-H and beta-I strands of VP3; this sequence in type 1 and type 2 poliovirions also contains a neutralizing epitope (Weigers & Dernick, 1992; Patel et al., 1993). The same structural region in the Mengo virion is represented by peptide K151, which has a high S.I. for TH cells from BALB/c (but not from SJL/J or C57BL/6) mice.

A number of previous studies have suggested that variable regions of protein pathogens are preferentially recognized by T cells. For example, examination of the normal T cell response to poliovirus in humans localized most of the response to VP1 to the hypervariable regions of the capsid protein (Graham et al., 1993). As well, analysis with poliovirus-specific murine CD4^+ T cell clones defined T cell epitopes in the variable region of VP1 adjacent to neutralizing antibody site I and in variable regions at the amino-terminal and in surface regions of VP3. Mutations within the T cell epitope have been shown to abolish virus-specific CD4^+ and CD8^+ T cell responses by two possible mechanisms (Mahon et al., 1992). First, recognition of the epitope by the MHC molecule or the T cell receptor can be affected by substitutions within the core of the epitope. Second, it is possible that substitutions in sequences flanking the core of the epitope prevent T cell recognition by altering the pattern of antigen processing. In our study a number of epitopes were identified in variable stretches on the interior and exterior surfaces of capsid proteins VP2 and VP3. The amino-terminal 14 residues of VP2 and 20 residues of VP3 contain TH epitopes. In addition, VP2 sequences represented by peptide K107 and parts of peptides K102 and K103 are composed of surface residues. Significant portions of peptides K130 and G135, which contain TH epitopes in VP3, are found on the surface. However, we (and Hastings et al., 1993) have identified epitopes in the two capsid proteins that are composed of interior residues in the protein tertiary structure, suggesting that T cell epitopes need not be confined to highly variable sites. Variation in the sequence of the internal structure of the capsid proteins cannot be ruled out as a mechanism for eluding immune surveillance, but these regions should be much less susceptible to sequence variation because of the requirement to maintain the structural and functional integrity of the capsid. Recognition of conserved regions by the T cells would be advantageous for the immune system because the virus would have only a very limited capacity to mutate these regions to avoid immune recognition. Additional support for this idea comes from the observation that VP4 of poliovirus, which is also found entirely on the interior of the capsid and is important for maintaining the structural integrity of the pentameric subunits of the capsid, is recognized by TH cells in both humans and mice (Mahon et al., 1992; Simons et al., 1993).

Examination of the Mengo virus TH cell epitopes detected in BALB/c mice and in two other strains of mice indicated that the pattern of recognition of these epitopes is dependent on the MHC haplotype (see Fig. 6). Of the 13 epitopes that induced a proliferative response in Mengo virus-primed LN cells from BALB/c (H-2^d) mice, 11 were recognized by similarly primed lymphocytes from C57BL/6 (H-2^b) mice, but only five were recognized in SJL/J (H-2^s) mice. The magnitude of the proliferative response induced by each peptide was also strain dependent. In fact, the response of SJL/J mice to whole inactivated virus, as well as most of the peptides, was consistently less than the response of BALB/c or C57BL/6 mice. This restriction of specificity is dependent on the nature of the peptide–MHC complex or of the T cell receptor (discussed in Panina-Bordignon et al., 1989). MHC molecules are highly polymorphic and studies have shown that a given peptide will generally bind to only one or a few alleles. However, the existence of promiscuous epitopes that are recognized by a large number of MHC class II molecule alleles has also been demonstrated (Panina-Bordignon et al., 1989). Five of the peptides shown to contain TH cell epitopes in this study (K89, K102, K113, K125 and G135) were recognized by all three strains of mice tested and may belong to the group of promiscuous epitopes. However, this would have to be tested on a large panel of mouse strains representing as many of the allelic forms of the MHC molecule as possible. Identification of such epitopes is important for understanding the restriction
Fig. 7. Comparison of the location of T cell epitopes predicted by the TSites AMPHI (■), Rothbard & Taylor motif ( ) and 'D' motif (□) algorithms with peptides containing the experimentally identified T cell sites (indicated above the sequence) in VP2 and VP3.

of T cell epitopes and could facilitate identification of similar epitopes in infectious organisms and help in the production of synthetic vaccines with broad specificity.

Considerable effort has been made to establish an *a priori* identification of immunogenic T cell epitopes, and we have examined the predictive power of the program TSites (Feller & de la Cruz, 1991) in the context of our experimental results for Mengo virus. The TSites program identified significant portions of all the capsid proteins as potentially antigenic based on selected criteria for the correlation of T cell antigenicity to protein sequence and structure. The number of predicted sites per unit length was roughly similar for capsid proteins VP1, 3 and 4. VP2, however, had a much higher number of epitopes predicted (particularly in the first 110 residues and the last 55 residues) by all strategies. This large number of predicted sites correlates well with the observed antigenicity of VP2 which contains 8 of the 13 experimentally identified peptides corresponding to sites of T cell immunogenicity.

In VP2 and VP3, 9 of the 13 peptides experimentally determined to contain $T_h$ epitopes overlap sites that were predicted to be potentially antigenic. No one algorithm of the program appeared to have any more significant success at predicting the sites than the others, and sites predicted by more than one strategy do not appear more likely to be real epitopes than sites predicted by only one algorithm. The fact that more of the predicted sites were not identified experimentally is not necessarily indicative of any lack of predictive power or specificity of the algorithms making up the TSites program because the limited scope of our experiments only identified peptides which were recognized by the H-2d MHC haplotype. It is more significant to note that the $T_h$ sites represented by
peptides K107 and K112 in VP2 and by peptides K124 and G135 in VP3 were not identified by any of the predictive strategies. Clearly there exist recognition motifs and/or structures that cannot yet be adequately predicted, even by a combination of searching strategies. Experimentally identified Tₜ epitopes in VP1 of picornavirus (Kutubuddin et al., 1992) have been compared with the AMPHI and the Rothbard & Taylor motif predictive strategies and no strong correlation was found between the predicted and observed epitopes. Studies with Dengue virus (LeClerc et al., 1993) and the Tₜ epitopes of lysozyme (Gammon et al., 1991) also demonstrate the difficulties of using the available predictive strategies to identify T cell epitopes. Experimental identification of epitopes currently appears to be the only reliable way to identify T cell epitopes recognized by specific class I or class II MHC molecules of various haplotypes. The identification and characterization of many more actual epitopes is required to refine predictive strategies.

We express our sincere thanks to Roger Bradley for technical assistance and Mike Mulvey for helpful comments. S.M. and D.K. both made significant contributions to this research. The studies were financed by a grant from the Medical Research Council of Canada to D.G.S.; S.M. is grateful to the Alberta Heritage Foundation for Medical Research for a Visiting Scientist Award, and D.K. holds an AHFMR Studentship.

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*(Received 5 April 1994; Accepted 15 June 1994)*