Superantigenicity of Streptococcal M Protein

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Summary

M proteins that define the serotypes of group A streptococci are powerful blastogens for human T lymphocytes. The mechanism by which they activate T cells was investigated and compared with the conventional T cell mitogen phytohemagglutinin, and the known superantigen staphylococcal enterotoxin B. Although major histocompatibility complex (MHC) class II molecules are required for presentation, there is no MHC restriction, since allogeneic class II molecules presented the bacterial protein to human T cells. Type 5 M protein appears to bind class II molecules on the antigen-presenting cells and stimulate T cells bearing Vβ 8 sequences. Our results indicate that this streptococcal M protein is a superantigen and suggest a possible mechanism of its role in the pathogenesis of the postinfectious autoimmune sequelae.

Infection with group A streptococci may evoke autoimmune diseases in the susceptible host that can affect the heart, kidney, or brain (1). The major virulence factor of these organisms appears to be the surface antigen M proteins, which are a family of closely related proteins emanating from the cell surface as α helical coiled coil fibrils (2). A number of studies have shown that purified streptococcal M proteins stimulate nonimmune human T cells to undergo brisk proliferation (3–6, and Kotb et al., manuscript submitted for publication). In contrast, synthetic peptides, up to 35 amino acids in length, copying various regions of the M protein, had no stimulatory effect. These studies suggested that M protein did not activate human T cells via classical T cell epitopes described for conventional antigens. Because of its effect on such large populations of T cells, M protein may be a superantigen (7), requiring larger domains of the protein to associate with class II MHC molecules and subsequent recognition by the TCR. To test this hypothesis, we conducted the following study to investigate the possible superantigenic properties of M protein.

Materials and Methods

Isolation and Purification of M Protein. M protein was purified by limited peptidase digestion of type 5 group A streptococci as described (5); the purified fragment is designated pep M5. Staphylococcal enterotoxin B (SEB) was from Sigma Chemical Co. (St. Louis, MO).

Isolation of Lymphocytes. PBMC were isolated from heparinized blood of a number of donors by Ficoll-Hypaque density gradient centrifugation (5). PBMC were further purified into T and B lymphocyte–enriched populations by two cycles of E rosetting (8), followed by overnight adherence of both populations at 37°C and 5% CO2 in RPMI 1640, 10% FCS, 2 mM l-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin (RPMI complete).

Lymphocyte Cultures for Assessment of T Cell Proliferation. PBMC (2 × 10⁶), or T cells (10⁵) along with 10⁴ irradiated B cells (3,000 rad), were incubated for 3 d (37°C, 95% humidity, and 5% CO2) with the indicated stimuli, and then proliferation was assessed by [³H]thyminine uptake as previously described (5).

mAbs. Hybridomas secreting anti-HLA-DR antibodies (L243), anti-HLA-DR,-DQ (9.3F10), were purchased from American Type Culture Collection (Rockville, MD) and purified over a protein A-sepharose column (Sigma Chemical Co.). Purified mAbs to a human HLA-A,B,C were from Cappel Laboratories (Malvern, PA). The mAbs to Vβ 5,6,8, and 12 were a generous gift from Dr. J.-C. Cerottini (Ludwig Institute for Cancer Research, Epalinges, Switzerland).

Transfected Mouse Fibroblasts. Mouse fibroblasts transfected with neomycin resistance gene only (L66), HLA-DR4:Dw14 (L165.1), HLA-DQ7a:DQw3β (L54.5), or HLA-DPw4a:DPw40 (L25.4) were a generous gift of Dr. Robert Karr, Iowa City, IA. These cells were maintained in the appropriate selection medium (DMEM; Gibco Laboratories, Grand Island, NY) containing 5 × 10⁻⁵ M 2-ME, 10% FCS, penicillin, streptomycin, 2 mM l-glutamine, and 0.25 mg/ml of G418 (Gibco Laboratories) as adherent lines in sterile tissue culture flasks. To assess their APC function, the cells were treated with 100 µg/ml mitomycin C for 1 h at 37°C, and then were added at 2 × 10⁵/well to purified T cells.

Binding of Pep M5 to HLA Class II⁺ Cells. Pep M5 (0.5 mg) in PBS (pH 7.2) was mixed with a 20-fold molar excess of biotin-X-NHS (Bio-Rad Laboratories, Richmond, CA) for 4 h at 25°C, dialyzed overnight against three changes of PBS (pH 7.2), and then filter sterilized. Purified T or B cells were incubated for 30 min with or without 5 µg/ml biotinylated pep M5, washed repeatedly, and incubated for 30 min with 50 µl (1:10 dilution) of FITC-conjugated avidin (Sigma Chemical Co.), and then analyzed using an Epics 753 dye-laser flow cytometer (Coulter Electronics Inc., Hialeah, FL) (5).
Results and Discussion

Pep M5 was used to stimulate highly purified human peripheral T cells in the presence or absence of accessory cells (AC). Although we previously showed that pep M5-induced T cell proliferation is AC dependent, the role of these cells in the response was not clear (4). Most antigens are processed by the AC and presented to the T cells in the context of MHC elements; whereas, mitogens require AC only to provide a solid matrix for crosslinking-relevant T cell surface molecules. To distinguish between these possibilities, we tested the presentation requirements for M protein. Unlike PHA, both M protein- and SEB-induced T cell stimulation require the expression of class II molecules by APC. AC lacking class II elements; whereas, mitogens require AC only to provide a necessary component(s) for stimulation by PHA (Fig. 1).

These results suggested that class II antigens are required for the presentation of pep M5. Despite this requirement, the presentation of pep M5 to T cells does not appear to be MHC restricted, since allogeneic cells were as effective as autologous cells in supporting human T cell proliferation in response to pep M5 (Table 1). The lack of MHC restriction was further shown in experiments where mouse L cells, transfected and expressing high densities of both HLA class II α and β chains, were used as AC. Fibroblasts transfected with HLA-DR, -DQ, and -DP were all capable of presenting pep M5 and to the known superantigen, SEB, but had little effect on the response to PHA (Fig. 1).

Table 1. MHC Nonrestricted Presentation of Pep M5 by APC

| Exp. | Donor APC | − Pep M5 | + Pep M5 |
|------|-----------|----------|----------|
| 1    | Autologous| 0.3 ± 0.04| 17.2 ± 1.0|
|      | Allogeneic| 0.6 ± 0.2 | 11.0 ± 1.0|
| 2    | Autologous| 1.2 ± 0.4 | 7.8 ± 0.3 |
|      | Allogeneic| 0.8 ± 0.04| 11.8 ± 0.7|
| 3    | Autologous| 2.0 ± 0.3 | 35.0 ± 1.6|
|      | Allogeneic| 6.0 ± 0.8 | 17.0 ± 1.4|
| 4    | Autologous| 0.4 ± 0.1 | 7.6 ± 0.5 |
|      | Allogeneic| 0.2 ± 0.1 | 5.0 ± 0.4 |

Results are representative of two different experiments, each was repeated at least three times with cells from various individuals. Proliferation was assessed after 3 d by [3H]thymidine uptake. Exps. 1 and 2 were conducted using T cell clones, whereas in experiments 3 and 4 freshly purified T cells were used.

Purified T cells (10^6) were incubated with 10^6 irradiated autologous or allogeneic B cells in the presence or absence of 5 μg/ml pep M5. Proliferation was assessed after 3 d by [3H]thymidine uptake. Exps. 1 and 2 were conducted using T cell clones, whereas in experiments 3 and 4 freshly purified T cells were used.

alone (L66) did not present pep M5 or SEB, but did provide the necessary component(s) for stimulation by PHA (Fig. 2A). Once again, antibodies to either HLA-DR, or HLA-DR, -DQ inhibited the presentation of M protein and SEB but did not significantly affect that induced by PHA (Fig. 2B). Our results suggest that M protein is neither a conventional antigen restricted by MHC elements, nor is it a typical mitogen, since class II-positive cells are required for its presentation to T cells.

Although the above data strongly suggest that pep M5 interacts with class II molecules, it was important to determine if we could detect physical binding between these molecules. To this end, biotinylated pep M5 was incubated with either purified T or B cells, and the binding was visualized using FITC-avidin and analyzed by flow cytometry. T cells were incapable of binding pep M5 (data not shown). As seen in Fig. 3, pep M5 bound avidly to B cells and, furthermore, this binding was reduced in the presence of either unbiotinylated pep M5 or anti-HLA-DR antibodies, but not by SEB, which also binds class II molecules (9–12, and our unpublished data). This is not surprising since SEB and pep M5 could be binding class II molecules at two distinct sites. Alternatively, pep M5 could be binding with a higher affinity than SEB, which would be analogous to previous observations showing that SEA can inhibit the binding of SEB to class II molecules but not vice versa (10). Antibodies to class II molecules were ineffective in competing for binding of biotinylated pep M5 to B cells (data not shown).

The superantigens described so far appear to interact with the human TC (α/β) receptor via a specific set of Vβ elements (13–16). To determine if T cell stimulation by pep M5 is Vβ specific, we analyzed the pep M5- and anti-CD3-stim-
Figure 3. Binding of pep M5 to HLA class II-positive cells. Purified T or B cells were incubated for 30 min with or without 5 μg/ml biotinylated pep M5. The cells were washed repeatedly and incubated for 30 min with 50 μl (1:10 dilution) of FITC-conjugated avidin. Background nonspecific fluorescence was detected in groups where FITC-labeled avidin was added alone and was subtracted from both the control (bold line) and the inhibited (thin line) values. The cells were analyzed by flow cytometry, and the data are representative of a series of three separate experiments. To control for nonspecific fluorescence, we tested the ability of various agents to compete for the binding by biotinylated pep M5: (A) competition with 50 μg unlabeled pep M5; (B) competition with 25 μg unlabeled SEB; and (C) competition with mAbs (6 μg protein) to HLA-DR. It should be noted that EBV-transformed B cells as well as transfected fibroblasts expressing HLA-DR, -DP, or -DQ bound pep M5, whereas cells from the human leukemic T cell line Jurkat were ineffective in this capacity (data not shown). This binding was also inhibited by unbiotinylated pep M5.

Table 2. Vβ-specific Stimulation of Human T Cells by Pep M5

| Stimulus | Vβ5 | Vβ6 | Vβ8 | Vβ12 | CD3 |
|----------|-----|-----|-----|------|-----|
| None     | 3.4 | 3.2 | 6.4 | 4.5  | 96.9|
| Anti-CD3 | 3.1 | 2.1 | 2.9 | 1.9  | 96.0|
| Pep M5   | 2.5 | 2.2 | 15.4| 3.1  | 98.3|
| SEB      | 4.1 | 3.6 | 2.5 | 5.0  | 97.3|

T cells (10⁶) and 5 x 10⁵ irradiated autologous B cells were cultivated in RPMI complete medium for 3 d in the presence or absence of 1 μg/ml pep M5, 10 μl anti-CD3 antibody, or 1 μg/ml SEB. After incubation, the cells were cultured for an additional 24 h in medium containing IL-2 to allow regeneration of potentially modulated receptors. Specific Vβ expression was determined using mAbs and indirect immunofluorescence. Analysis was performed using flow cytometry, gating on the large blasted cells.
icry between the M protein and host tissue proteins, and that humoral immunity may be involved in this process. Based on our new findings, our views may need to be modified to envision M protein as a superantigen that may stimulate subsets of T cells with specificity for self antigens (e.g., cardiac tissue) above a threshold needed to arouse an autoimmune response.

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