

## **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 V $\beta$  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  $\alpha$  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 pepsin 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% CO<sub>2</sub> in RPMI 1640, 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50  $\mu$ g/ml streptomycin (RPMI complete).

**Lymphocyte Cultures for Assessment of T Cell Proliferation.** PBMC ( $2 \times 10^6$ ), or T cells ( $10^5$ ) along with  $10^5$  irradiated B cells (3,000 rad), were incubated for 3 d (37°C, 95% humidity, and 5% CO<sub>2</sub>) with the indicated stimuli, and then proliferation was assessed by [<sup>3</sup>H]thymidine uptake as previously described (5).

**mAbs.** Hybridomas secreting anti-HLA-DR antibodies (L243), anti-HLA-DR, -DQ (9.3 F10), 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 V $\beta$  5,6,8, and 12 were a generous gift from Dr. J.-C. Cerottini (Ludwig Institute for Cancer Research, Epalinges, Switzerland).

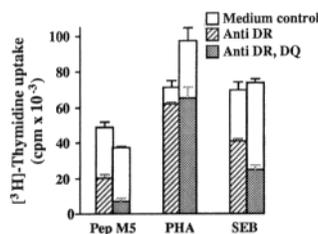
**Transfected Mouse Fibroblasts.** Mouse fibroblasts transfected with neomycin resistance gene only (L66), HLA-DR4:Dw14 (L165.1), HLA-DQ7 $\alpha$ :DQw3 $\beta$  (L54.5), or HLA-DPw4 $\alpha$ :DPw4 $\beta$  (L25.4) were a generous gift of Dr. Robert Karr, Iowa City, IA. These cells were maintained in the appropriate selection medium (DMEM; Gibco Laboratories, Grand Island, NY) containing  $5 \times 10^{-5}$  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  $\mu$ g/ml mitomycin C for 1 h at 37°C, and then were added at  $2 \times 10^4$ /well to purified T cells.

**Binding of Pep M5 to HLA Class II<sup>+</sup> 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  $\mu$ g/ml biotinylated pep M5, washed repeatedly, and incubated for 30 min with 50  $\mu$ l (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 molecules, such as U937 cells and human foreskin fibroblasts, were ineffective in presenting pep M5 to T lymphocytes (data not shown). Because these observations suggested that pep M5 may associate directly with class II molecules, we asked if antibodies to these molecules would interfere with this association. Monoclonal anti-HLA-DR and anti-HLA-DQ inhibited the response of human T lymphocytes to pep M5 and to the known superantigen, SEB, but had little effect on the response to 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  $\alpha$  and  $\beta$  chains, were used as APC. Fibroblasts transfected with HLA-DR, -DQ, and -DP were all capable of presenting pep M5 and SEB to purified human T lymphocytes. In contrast, fibroblasts transfected with the neomycin resistance gene



**Figure 1.** Inhibition of pep M5-induced T cell proliferation by anti-HLA class II antibodies. PBMC were stimulated with 5  $\mu$ g/ml pep M5 or PHA, or 1  $\mu$ g/ml SEB. Monoclonal anti-HLA-DR, and -HLA-DQ were added to the cultures, as indicated, at 12  $\mu$ g protein/culture. Proliferation was assessed after 3 d by measuring [<sup>3</sup>H]thymidine uptake. In the absence of either stimulants or antibodies, uptake values were 3.8 and 3.9 for each experiment, and in the presence of anti-HLA antibodies, these values were 2.6 and 2.4, respectively. To control for nonspecific inhibition, we used a similarly purified monoclonal anti-S-adenosylmethionine synthetase that had no significant inhibitory effect on either pep M5 or SEB stimulation. Results are representative of two different experiments, each was repeated at least three times with cells from various individuals. Inhibition was also observed with antibody concentrations of 15, 6, and 3  $\mu$ g protein/culture.

**Table 1.** MHC Nonrestricted Presentation of Pep M5 by APC

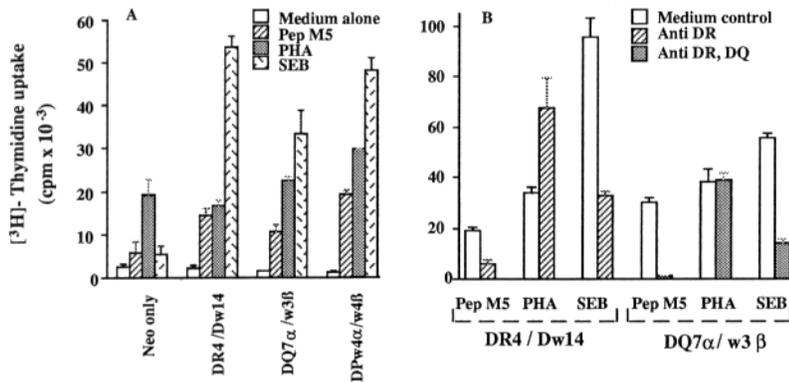
Exp.	Donor APC	[ <sup>3</sup> H]Thymidine uptake	
		- Pep M5	+ Pep M5
		<i>cpm</i> $\times$ 10 <sup>-3</sup> $\pm$ SEM	
1	Autologous	0.3 $\pm$ 0.04	17.2 $\pm$ 1.0
	Allogeneic	0.6 $\pm$ 0.2	11.0 $\pm$ 1.0
2	Autologous	1.2 $\pm$ 0.4	7.8 $\pm$ 0.3
	Allogeneic	0.8 $\pm$ 0.04	11.8 $\pm$ 0.7
3	Autologous	2.0 $\pm$ 0.3	35.0 $\pm$ 1.6
	Allogeneic	6.0 $\pm$ 0.8	17.0 $\pm$ 1.4
4	Autologous	0.4 $\pm$ 0.1	7.6 $\pm$ 0.5
	Allogeneic	0.2 $\pm$ 0.1	5.0 $\pm$ 0.4

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

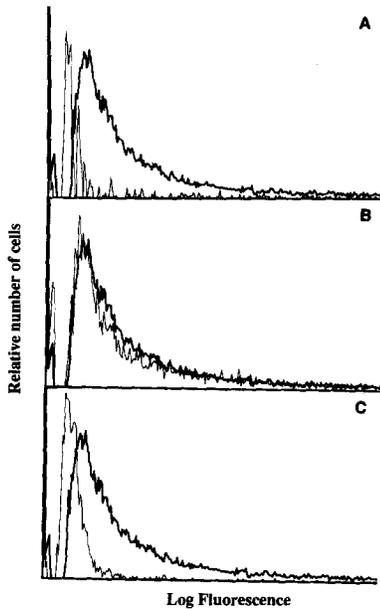
alone (L66) did not present pep M5 or SEB, but did provide the necessary component(s) for stimulation by PHA (Fig. 2 A). Once again, antibodies to either HLA-DR or HLA-DQ inhibited the presentation of M protein and SEB but did not significantly affect that induced by PHA (Fig. 2 B). Our results suggest 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 I 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 ( $\alpha/\beta$ ) receptor via a specific set of V $\beta$  elements (13-16). To determine if T cell stimulation by pep M5 is V $\beta$  specific, we analyzed the pep M5- and anti-CD3-stim-



**Figure 2.** Presentation of pep M5 by HLA class II-transfected mouse fibroblasts. T cells ( $10^5$ ) were cultured with or without  $2 \times 10^4$  mitomycin C-treated mouse fibroblasts transfected with neomycin resistance gene only (L66), HLA-DR (L165.1), HLA-DQ (L54.5), or HLA-DP(L25.4). (A) Cultures were incubated for 3 d in medium alone or in the presence of pep M5 (5  $\mu\text{g}/\text{ml}$ ), PHA (5  $\mu\text{g}/\text{ml}$ ), or SEB (1  $\mu\text{g}/\text{ml}$ ). Proliferation was assessed by [ $^3\text{H}$ ]thymidine incorporation. The data are representative of a series of five experiments and are expressed as the mean of triplicate cultures  $\pm$  SEM. (B) Purified monoclonal anti-DR, or anti-DR, -DQ were included in the culture, as indicated above, at 6  $\mu\text{g}$  protein/well. The data represent one of three experiments and show typical results. In B, L66 (Neo only) did not present pep M5 or SEB, while supporting the response to PHA with uptake values of 41.5 and 47 in the absence and presence of anti HLA-DR, respectively.



**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  $\mu\text{g}/\text{ml}$  biotinylated pep M5. The cells were washed repeatedly and incubated for 30 min with 50  $\mu\text{l}$  (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  $\mu\text{g}$  unlabeled pep M5; (B) competition with 25  $\mu\text{g}$  unlabeled SEB; and (C) competition with mAb (6  $\mu\text{g}$  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.

ulated blast cells for expression of V $\beta$  5, 6, 8, or 12 by flow cytometry. A significant expansion of V $\beta$  8-bearing T cells was observed (Table 2), but due to the limited mAbs to specific V $\beta$ s, it was not possible to determine if other V $\beta$  elements were also expanded. We have recently adopted the technique of Choi et al. (16), using PCR and specific primers to the various V $\beta$  families. Preliminary experiments confirm the usage of V $\beta$  8 and indicate the usage of a set of additional V $\beta$ s.

Based on the above findings, we propose that streptococcal M protein is a superantigen, resembling a number of staphylococcal toxins (7, 15, 16) and a soluble product secreted by *mycoplasma arthritidis* (17). Marrack, Kappler, and colleagues (7) have suggested a role for superantigens in the development of autoimmune disorders, and various enterotoxins have been implicated in this respect. It is well known that autoimmune disorders such as rheumatic heart disease and glomerulonephritis may occur after infection with group A streptococci. Our thinking for some time has been that these autoimmune diseases may be the result of molecular mim-

**Table 2.** V $\beta$ -specific Stimulation of Human T Cells by Pep M5

Stimulus	V $\beta$ 5	V $\beta$ 6	V $\beta$ 8	V $\beta$ 12	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 ( $10^6$ ) and  $5 \times 10^5$  irradiated autologous B cells were incubated in RPMI complete medium for 3 d in the presence or absence of 1  $\mu\text{g}/\text{ml}$  pep M5, 10  $\mu\text{l}$  anti-CD3 antibody, or 1  $\mu\text{g}/\text{ml}$  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 V $\beta$  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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