NONSPECIFIC COMPLEMENT ACTIVATION BY
STREPTOCOCCAL STRUCTURES

I. Re-Evaluation of HLA* Cytotoxicity Inhibition†

BY J. W. TAUBER, J. A. FALK, R. E. FALK, AND J. B. ZABRISKIE

(From The Rockefeller University, New York 10021; the Tissue Typing Laboratory, Toronto Western Hospital; and the Department of Surgery and Institute of Immunology, University of Toronto, Ontario, Canada)

Cross-reactions between mammalian transplantation antigens and certain bacterial structures have been postulated on the basis of various observations. In a series of convincing experiments, Rapaport and Chase (1-3) have clearly demonstrated that skin graft sensitization may be achieved with streptococcal membrane antigens in a variety of different species, such as guinea pigs, mice, rats, or rabbits (4). However, it has been difficult to determine from these experiments whether the observed sensitization was related to antigenic similarities between the bacterial membranes and organ-related transplantation antigens or to products of the major histocompatibility complex (HLA) itself. Therefore, the intriguing question remained: namely, did cross-reactivity between streptococcal antigens and mammalian tissues extend to the individual HLA antigens? The only direct evidence for this possibility has come from HLA cytotoxicity inhibition studies with bacterial structures. In spite of substantial efforts in this direction (5), such studies have remained ambiguous. For example, nonserological inhibitory mechanisms have been suggested (6) while in other experiments specific antibody other than anti-HLA was not strictly excluded (7). In view of these discrepancies, the true nature of the observed inhibition phenomena had to be re-examined before any conclusions about antigenic relationships between histocompatibility antigens and bacterial structures could be drawn. For this purpose, the HLA studies were repeated with the proper controls. The present report indicates that there is no evidence for a serological cross-reaction between streptococcal membrane antigens and the HLA antigens. Rather, the observed inhibitory effect appears to be based on a nonspecific anticomplementary activity of these bacterial structures.

Materials and Methods

Bacterial Strains. Throughout these experiments, we used two strains of streptococci from the collection of Dr. R. C. Lancefield. Strain 643/122/2 is a Group A serological type 6 originally isolated in 1919 from patient No. 43 during the epidemic outbreak at Fort Houston, San Antonio, * Nomenclature according to WHO-IUIS Terminology Committee Nomenclature for Factors of the HLA Systems. 1976. Transplant. Proc. 8:109.
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Texas, and has been cultured through 192 mouse passages. The second strain, A832, is a Group D streptococcus originally isolated from neonatal blood in 1967 during an outbreak of streptococcal infections in the nursery of Lincoln Hospital, New York. The two strains were kindly made available in lyophilized form by Dr. R. C. Lancefield, The Rockefeller University, New York.

Preparation of Media and Growth. Lyophilized streptococci were resuspended in 5 ml of Todd-Hewitt broth (8) to which 0.2 ml of defibrinated normal rabbit blood has been added. After an incubation of 24 h at 37°C, the microorganism was transferred to 30 ml of Todd-Hewitt broth, then 250 ml, and finally, 20 liters of final medium (9). The final medium was prepared as follows: for each 20 liters of medium, 600 g of yeast extract (Difco Laboratories, Detroit, Mich.) and 60 g of glucose were mixed with 600 cm³ of H₂O and heated to 80°C for 15 min with constant stirring. 1 cm³ of an antifoaming agent (Dow Corning antifoam compound; Dow Corning Corp., Midland, Mich.) was also added to prevent excessive foaming. After cooling, the contents were placed in dialysis sacks (catalogue no. 8-667C; Fisher Scientific Co., Pittsburgh, Pa.). Care was taken not to fill the dialysis sacks beyond half volume. The sacks were dialyzed against two changes of 10 liters of distilled H₂O at 4°C. The dialysate material was adjusted to 20 liters and filtered through an FP-1616 Selas-type filter candle, porcelain, 02 porosity (SGA Scientific, Inc., Bloomfield, N. J.).

Purity of the organism was checked at each step of the procedure by streaking the inoculum on sheep bleed agar plates. After 18 h of incubation at 37°C, the cells were harvested in a Sharples continuous flow centrifuge (Sharples-Stokes Div., Penwalt Corp., Warminster, Pa.).

Preparation of Pure Membranes. The cell pellet was washed once in 300 ml phosphate-buffered saline (PBS)¹ and then taken up in 0.05 M phosphate buffer, pH 6.1, containing 4% NaCl and 0.005 M EDTA with activated phage-associated lysin and incubated at 37°C for 2 h (10). This enzyme, an L-alanine-amidase, breaks the peptide bridge responsible for cross-linking the peptidoglycan moiety, leaving the fragile protoplasts intact. The protoplasts were centrifuged down at 4,000 g, washed three times in isotonic saline, and resuspended in 0.02 M phosphate buffer, pH 7.3, containing 0.02% NaCl and 0.1% MgCl₂, 6H₂O with 20 mg DNase and RNase per liter, and incubated for 1 h at 37°C. This hypotonic buffer ruptured the protoplasts, permitting release of the intracellular contents. The naked membranes were separated from the cytoplasmic components with high speed centrifugation at 27,000 g and again incubated with the RNase and DNase buffer. The membrane pellet was washed five times in 200 ml of PBS and followed by an equal number of washes in distilled water. The final wash in distilled H₂O was followed by resuspension of the membranes in 200 ml of distilled water and an aliquot removed and lyophilized to determine the concentration of the membranes. The suspension was frozen in aliquots at -70°C until use.

Special concern was given to the technique of resuspension during the washes. This seems to be an important detail to achieve pure preparations, since the membrane pellets have a strong tendency to adhere to each other during centrifugation procedures. For this purpose, an ultrasonic water bath (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) was employed. The water level was carefully adjusted so that maximum activity occurred in a single central spot. The membrane suspension was placed in the sonic field for 5–20 min to achieve a homogeneous suspension during each wash.

The enzymatic separation of wall and membrane could not be used for the Group D membranes since the phage-associated lysin is restricted in its specificity. Therefore, the Group D streptococci were mechanically disrupted using a Braun disintegrator (B. Braun Instruments, San Mateo, Calif.) (11). The disruption was monitored by gram stain which showed loss of the gram-positive staining in 90% of the cells after 1 h of treatment in the disintegrator. After differential centrifugation to isolate the cell walls and membranes, both materials were treated with RNase buffers as described above.

Because of difficulties inherent in separating cellular components completely by mechanical means, the resulting membrane preparation was not as pure as the enzyme-induced material. The biochemical distribution of rhamnose according to Dische and Shettles (12) provides evidence for the degree of purity:

|       | A          | D          |
|-------|------------|------------|
| content of rhamnose (%) | 0.5        | 4.1–5.9    |
| contamination with wall (%) | 1.9        | ?          |

¹ Abbreviation used in this paper: PBS, phosphate-buffered saline.
Rhamnose is the major sugar moiety and composes 27% of the weight of the cell wall of Group A streptococci; it is not present in pure membrane preparations (13) in Groups A and C.

The lyophilized membranes were used in various modifications to absorb out HL-A antisera. After assessment of the dry weight, membranes were suspended in serum-free McCoy's medium (catalogue no. 166-O; Grand Island Biological Co., Grand Island, N. Y.). To achieve homogeneous dispersion, this solution was sonicated for 30 s while submerged in an ice bath. A Sonifier Cell Disruptor, Model W185 (Heat Systems-Ultrasoundics, Inc.) with a regular probe was used for this purpose, and the output control was set on position 5. The milky suspension was mixed with an equal volume of McCoy's medium containing 30% fetal calf serum. Further dilutions were made with 15% serum-containing McCoy's medium.

These suspensions were incubated immediately with the specific antisera to be tested. Immediate incubation was felt to be crucial for the absorption studies in order to prevent reaggregation of membrane fragments which might mask important antigenic determinants. Thus, the delay from sonication to incubation with the HL-A antisera never exceeded 2 min.

**Tissue Typing.** Tissue typing was carried out in Terasaki trays (14), and for special studies the Scandinavian method was applied in parallel (15). Briefly, the following quantities of materials were used: 1 µl of specific HLA antiserum of a given dilution was introduced into the microwell with a Hamilton syringe (Hamilton Co., Reno, Nev.). If an inhibition protocol was followed, then 1 µl of just-sonicated membranes was added into this same well as the second step. After an incubation of 30 min, 1 µl of the target cells containing 2 × 10⁶ lymphocytes/ml was added to the tray. After an additional incubation at 37°C for 30 min, 5 µl of complement (C) was added. The amount of membranes used is given as the final concentration in the total vol of the microwell which is 8 µl. After an additional 1 h at room temperature, 3 µl of an aqueous solution of 5% eosin Y (Tetrabromofluorescinsodium; Fisher Scientific, Inc.) were added, and the percent of dead cells estimated under the microscope. Further modifications of the procedure were outlined with the corresponding results.

**HLA Antisera.** The panel of antisera of the Tissue Typing Laboratory, Toronto Western Hospital, Toronto, Canada, was screened for four representative specificities of the major "public" families of histocompatibility antigens. These sera were carefully selected in order to avoid cross-reactivity within the families of HLA antisera (Table I). The selected antisera detected the following HLA antigens: A1 and A2 from the first series, and B7 and B12 from the second series. To confirm that any specific results would be due to the HLA antibody and not to other unknown constituents of a given antiserum, three different sources with identical A2 specificities were compared. One of these antisera was a purified myeloma protein of the IgG2 class with functional activity specific to the A2 antigen (16). The activity, strength, and optimal titers of the antisera used in these studies were determined in preliminary trials and will be indicated for each set of experiments.

**Complement.** Blood was obtained from 3-wk-old rabbits; the serum was separated and stored away in aliquots at −70°C. Undiluted serum freshly thawed was used in all experiments.

**Target Cells.** Lymphocytes from three different donors were employed and represented one or more of the histocompatibility antigens detected by the antisera. Peripheral blood was freshly collected and defibrinated with glass beads. The lymphocytes were then selectively harvested by floating on a Ficoll-Hypaque solution (1.080 g/ml) (17). If the number of polymorphonuclear leukocytes in the mononuclear layer was too high, further purification was achieved by the addition of carbonyl iron to the cells (18) followed by magnetic removal of those polymorphonuclear neutrophils which had ingested the carbonyl iron. To avoid any peculiarities of the donor, various donor sources representative for identical specificities were used in all experiments.

**Results**

Because of the complexity of the cytotoxic assay system itself, it was decided to test the individual components of the system in a series of experiments. The results of these experiments will be reported under separate headings, together with the additional technical details involved in each experiment.

**Confirmation of Inhibitory Effect.** The purpose of these experiments was to confirm the findings of previous authors, namely, that streptococcal antigens
can indeed inhibit the cytotoxic effect of HLA antisera. Thus, the standard procedure of these earlier reports was followed as closely as possible (5, 7). Briefly, the undiluted HLA antisera were distributed into the Terasaki wells (14), as well as Scandinavian trays (15). Then, the freshly sonicated membranes of Group A and Group D streptococci were added to the wells at different concentrations. After incubation of 30 min at 37°C, the target cells were added, followed by the addition of C as described in previous sections.

The results are summarized in Table II and clearly confirm the previous observations. All HLA antisera were successfully inhibited, regardless of their specificity, as long as adequate amounts of membranes of Group A streptococci had been added. In contrast, membranes from Group D streptococci were much less efficient. Inhibition was seen with only one B12 donor and these experiments could not be reproduced with target cells of another similar donor. In general, the results of the Scandinavian method did not differ materially from the results obtained with the Terasaki method.

These findings clearly raised the suspicion that nonspecific inhibitory mechanisms might be operative. Fig. 1 gives a schematic view of the theoretical possibilities. For expression of direct cytotoxicity the three elements, target cell, specific antibody, and C have to be granted free interaction under suitable conditions. Any interference between these three elements can lead to abrogation of the cytotoxic effect. We decided to investigate the two most likely nonspecific mechanisms of inhibition. First, evidence by other investigators had suggested that streptococcal membrane fragments adhered to the surface of lymphocytes, thereby masking or sterically blocking HLA receptors (6). To test this theory, we modified the procedure as follows: The target cells were incubated with antisera before addition of streptococcal membranes and C. It was hoped that the blocking effect could be circumvented by this technique. If so, the theory of the surface effect would become the most acceptable.

The second hypothesis involves the possibility of blocking cytotoxicity via nonspecific mechanisms, namely, the inactivation of C. This phenomenon is
TABLE II

| Antiserum          | A1 | A2 | A2 | A2 | B7 | B12 |
|--------------------|----|----|----|----|----|-----|
| Lymphocyte donor   | ○  | △  | △  | △  | △  | △   |
| Streptococcal      | None | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| membranes (1.3mg/ml) | Group A | −  | −  | −  | −  | +  | −  | −  | −  | −  | −  | −  |
|                    | Group D | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | −  | ++++ |

○, A1, B7; △, A1, B7, B12; ○, A2, A9, B12; ++++, all cells dead; and −, all cells alive.

![Diagram]

**Fig. 1.** A schematic representation of the possible sites of cytotoxicity inhibition by bacterial antigens. Each arrow includes two alternatives. The interference between antibody (Ab) and target cell could occur at either the cell surface or at the antibody-combining site. The interference between C and antibody could be achieved by the direct attack of C or the blocking of the Fc portion of the antibody. Ly, lymphocyte.

well known to serologists and has been observed with a variety of antigens, in particular with tissue extracts. To investigate this possibility, we decided to further modify our test system rather than to measure C consumption directly. Thus, we maintained the cytotoxicity system as such, but started with incubation of C and streptococcal membranes before their addition to the cells and antisera. The inhibitory effect was expected to be accentuated under these conditions since streptococcal membranes could interact with all available C before the introduction of competing cytotoxic immune complexes. The standard procedure and the above two modifications were carried out simultaneously and are reported as our second series.

**Determination of Inhibitory Mechanisms.** The purpose of these experiments was to differentiate between various nonspecific mechanisms of cytotoxicity inhibition. An exact outline of the suitably adapted modifications of the procedures is given in Table III. Special attention was given to the exact timing of the addition of each component of the cytotoxic assay. The moment of adding the membranes to the various other components was felt to be crucial and thus synchronized as 0. This was done with the intention of avoiding any delay between sonication of the membranes and assay of their activity. Thus, any observed difference could not be attributed to reaggregation of membrane fragments during waiting periods with consequent loss of activity.

Three HLA antisera were tested with these three procedures. Doubling dilu-
Table III
Time Schedule and Modifications in the Sequence of Addition of Various Components in Cytotoxicity Inhibition Studies

| Hours | I       | II      | III     |
|-------|---------|---------|---------|
| -1    | HLA-Ab* | HLA-Ab  | C       |
| 0     | Membrane§| Ly‡     | Ly      |
| 1     | Ly      | C       | Combined|
| 2     | C       | Read    |         |
| 3     | Read    |         |         |

* HLA-Ab, HLA antibody.
‡ Ly, lymphocytes.
§ Membrane, streptococcal membranes.

...tions were made up for this purpose reaching the end point of toxicity at titers of 1:256. Each procedure was carried out with membranes of Group A and Group D, as well as normal saline as a proper control. The results of these experiments are depicted in Fig. 2. While all experiments were carried out with membrane concentrations ranging from 7 to 250 µg/ml, for the sake of clarity only two concentrations of membranes and one HLA antiserum (B7) were selected for depiction in the graphs. The three columns refer to the three procedures as outlined on the top of the graphs (see Table III for details). The horizontal rows illustrate the experiments with the two concentrations of membrane (30 and 60 µg indicated on the right side of the graph). Each individual graph gives the percent of dead cells on the ordinate and dilution of the antiserum as a reciprocal on the abscissa. The stippled area represents the control run with normal saline, while the solid line gives the data after the addition of Group A membranes. The broken line refers to the effect produced by Group D membranes.

If we consider the first column where the standard procedure was followed (I) in which antibody were incubated first with membranes, we can recognize that toxicity is neutralized at a titer of 1:32 with 60 µg/ml membranes of Group A streptococci. Of interest was the consistent finding that Group D membranes appeared to enhance cytotoxicity rather than exerting a blocking effect. All other concentrations of Group A membranes (from 7 to 250 µg/ml) did not affect the cytotoxic reaction and indicated a rather narrow range of the membrane inhibitory effect. The dilution of the antiserum was also crucial. Undiluted antisera did not emphasize the inhibitory effect of the membranes, while a 1:32 dilution of the serum was completely inhibited. The inhibitory effect was lost with further dilutions, primarily on the basis of loss of cytotoxicity of these weak HLA antisera. These variations only serve to underscore the value of testing all combinations of antisera dilutions and membrane concentrations.

The second column evaluates the surface effect of streptococcal membranes. As indicated at the top of column II, antibody and target cells were mixed before streptococcal membranes would have a chance to interfere with the reaction. Inhibition of cytotoxicity thus would be circumvented if the surface hypothesis were true. Quite in contrast, we see now that both Group A, as well as Group D...
membranes, are efficient blockers of cytotoxicity. This renders the theory of a surface effect untenable.

In the third column, the anticomplementary hypothesis is tested. C and streptococcal membranes were incubated ahead of time as indicated on the top of this column. Here we can readily see the marked inhibitory effect of the membranes on the cytotoxic assay. These results clearly favor the anticomplementary mechanism. At first glance, these results appear to be contradictory to those obtained when Group A and Group D membranes were mixed with undiluted HLA antisera of known specificities (see Table II). In the first set of experiments, Group D membranes even at a concentration of 1.3 mg had no inhibitory effect on the cytotoxicity of known HLA antisera. However the conditions of the two experiments were radically different. In the former tests undiluted antisera was used and a single concentration of membranes employed, while in the present set of experiments different concentrations of both membranes and antisera were combined in the system. Thus we can conclude that at certain concentrations of membrane and dilutions of serum, both Group A and Group D membranes are effective inhibitors of the cytotoxicity test. However Group A membranes at all concentrations were more effective inhibitors of the system.

Search for Specific Serological HLA Inhibition Unrelated to C Consumption. With the observation that the inhibitor of cytotoxicity was due to disturbance in C activity, a search was made to circumvent the C effect. Accordingly, experiments were designed to test the absorption effect of streptococcal membranes with HLA antisera before the addition of C to the assay system. It was therefore decided to absorb the HLA antisera first with these bacterial membranes. After high speed centrifugation to eliminate all membrane fragments, the clear supernates could be tested for remaining toxicity. In detail, the
following protocol was observed: HLA antisera were incubated with Group A and Group D membranes, respectively, at 100 μg/ml for 30 min at 37°C and in the cold overnight. The following day, the suspensions were centrifuged at 3,000 g for 1 h in the cold and the supernates saved. A saline control was included for each set of experiments. All HLA antisera still exhibited specific toxicity against the appropriate target cells. Thus, no inhibition of HLA cytotoxicity was observed when streptococcal membranes were incubated and then removed from HLA antisera.

Cytotoxicity Experiments with Group A Membrane Antisera. While our experiments failed to uncover any relationship between these streptococcal antigens and the known HLA antigens, it was still conceivable that the presumed antigenic relationship was concerned with either HLA A or B of yet unknown specificity or to other gene products (HLA D) of the major histocompatibility complex. It was therefore decided to test cross-reactivity via a reverse route: namely, to use antisera raised against streptococcal membranes in the classical cytotoxicity experiments. Accordingly, rabbits were immunized with membranes isolated from Group A streptococcal strain T12/126/6. These sera were shown to brightly stain sacoemmal membranes by the indirect fluorescence technique (19). Such sera were then tested for cytotoxicity in a final dilution of 1:2 with PBS using the Terasaki procedure (14) (kindly carried out by M. Fotino, Tissue Typing Laboratory, New York Blood Center, New York). Human lymphocytes from all HLA types and all major W specificities were used as target cells (Table IV). The results of these efforts clearly demonstrated that no toxicity was exerted by our antisera regardless of the specificities of target cells. In addition, utilizing the indirect fluorescence technique, no selective lymphocyte staining was achieved with antistreptococcal membrane antisera.

Discussion
The inhibition of cytotoxicity of HLA antisera observed in these studies is evidently not due to specific cross-reactions between HLA antigens and streptococcal membranes, but rather to the anticomplementary activity of the latter. How do our findings fit with the data reported by Hirata and Terasaki (5)? They have shown that extracts of type 1 M-protein inhibit HLA antisera specific to A1, A2, A3, A9, B7, and B8. Incubation of C with this soluble test substance alone for 1 h before the addition of the lymphocytes decreased cytotoxicity by 20% only. These authors concluded from these experiments that an anticomplementary effect of the test substance could not account for the observed inhibition. However, their preincubation period is in a completely soluble system and is therefore not strictly comparable to the cytotoxicity assay where we always have a solid phase secondary to the introduction of lymphocytes. Thus, the activity of these soluble extracts in the presence of a membrane surface (i.e., the lymphocyte membrane) cannot be excluded and remains a distinct possibility. Moreover, the formation of immune complexes of M1 protein with antibody other than HLA antibody has not been excluded and could be responsible for the consumption of C with the corresponding nonspecific abolition of cytotoxicity. It has since been confirmed (20) that a variety of gram-positive bacterial structures share such anticomplementary activity. In a subsequent report (20), this anti-
TABLE IV

|                  | First series | Second series |
|------------------|--------------|---------------|
| Mixture I        | A11, AW33, AW25 | B13, B18, B27 |
| Mixture II       | A2, A3       | B7, B8, B12, BW15 |
| Mixture III      | A1, A2       | B12, BW19, B14, BW17 |
| Mixture IV       | A9, AW30     | B12, BW5, B18, BW22 |

complementary activity has been identified as part of the alternate pathway. Factor D seems to play the key role in this activation, while neither properdin nor antibody are required. With respect to the data of Rapaport et al. (6), these authors have already suggested that nonserological, nonspecific mechanisms may be responsible for the observed cytotoxicity inhibition. However, they attribute the inhibition to binding of their material to the lymphocyte surface, thus sterically hindering cytotoxic effects of HLA antisera. They also were unable to demonstrate an anticomplementary effect of their streptococcal preparation but the use of another soluble substance raises the same questions presented by Hirata's experiments (see above).

Unfortunately, the lack of evidence for specific cross-reactions between HLA antigens and streptococcal membranes is, in reality, a negative result and only seems to emphasize the disadvantages of such findings. It remains impossible to exclude such relationships entirely, moreover, because not all HLA specificities were tested and not all specificities are known. Indeed, numerous closely related products of the remaining major histocompatibility complex would have to be taken into consideration as well. However, these products are even less well defined and specific antisera are not yet available for all of these markers.

Similar results were seen in the last series of experiments, in that we were unable to demonstrate cross-reactions using antistreptococcal membrane antisera and a panel of lymphocytes. At least two interpretations of these results are possible. First, in spite of the screening type of analysis used, we might have missed the appropriate specificity of the target cells again. Secondly, bacterial membranes are heterogeneous antigens, and the antibody might have been formed predominantly against haptenic determinants other than the ones supposed to relate to HLA antigens.

We have formulated a model of the immune system² which suggests that bacterial antigens would not (except under the rarest circumstances) be cross-reactive with our HLA antigens, and the findings reported here are at least compatible with this concept. This does not, however, detract from the bacterially induced homograft sensitization experiments of Rapaport and Chase (1), since this phenomenon could be due to shared antigens related to the graft organ rather than the HLA antigens. In fact, mammalian antigens successfully mimicked by bacteria, such as Group A streptococci, have all been shown to be organ related (22). From this, we can speculate that HLA markers might have evolved

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² Tauber, J., E. C. Gotschlich, and J. B. Zabriskie. The T-cell receptor: evolution, supramolecular organization, and general principles of recognition. Manuscript in preparation.
through selective evolutionary pressure as an escape from microbial mimicry. These markers would be characterized by a high genetic polymorphism so that their mimicry would fail to provide an epidemiological advantage for the invading organism. A more detailed discussion of these general principles and concepts has been submitted elsewhere.

**Summary**

A number of experiments have suggested that there is an antigenic relationship between the HLA complex and streptococcal bacterial structures. Using inhibition of cytotoxicity of HLA antisera as our assay system, it was demonstrated that the inhibitory effect on HLA cytotoxicity by streptococcal antigens is, in reality, due to activation and consumption of components of the alternate complement pathway. In addition, antisera prepared against streptococcal membrane antigens had no cytotoxic effect on a large panel of human lymphocytes, nor did these antisera exhibit immunofluorescent staining of lymphocytes directly. These experiments are compatible with our concept that the HLA complex may have evolved through selective evolutionary pressure as a means of escaping bacterial mimicry.

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