ASSOCIATION OF HL-A 5 AND IMMUNE RESPONSIVENESS IN VITRO TO STREPTOCOCCAL ANTIGENS*

By LEONARD J. GREENBERG, ERNEST D. GRAY, AND EDMOND J. YUNIS

(From the University of Minnesota Hospitals, Department of Laboratory Medicine and Pathology, and the Department of Pediatrics and Biochemistry, Minneapolis, Minnesota 55455)

Studies in mice have provided data linking immune responsiveness (1–3), autoimmune disease (4), and resistance or susceptibility to virus infection and neoplasia (5, 6) to the murine major transplantation (*H-2*) antigen complex. The association between a transplantation antigen, a specific antibody response, and severity of disease observed in the case of murine autoimmune thyroiditis (4) has given credence to the notion that histocompatibility-linked immune response genes may play an important role not only in resistance or susceptibility to autoimmune disease, but also to neoplasia and disease in general.

There studies have led to the presumption that the observed associations are meaningful for survival and therefore have been preserved in evolution. As a consequence, many investigators have searched for association between human histocompatibility antigens (HL-A) and susceptibility or resistance to specific diseases. Several examples of HL-A-associated diseases have been described: the 4C complex and Hodgkin's (7–10), HL-A2-12 in acute leukemia and lymphoma (10–14), W15 in systemic lupus erythematosus (15, 16), HL-A 1–8 in chronic hepatitis (17), HL-A 3–7 in multiple sclerosis (18), W17 in psoriasis (19, 20), and W27 in Reiter's syndrome and ankylosing spondylitis (21–23). In the case of Hodgkin's disease and acute leukemia and lymphoma the correlations vary from population to population and are negative in some cases. Furthermore, in those populations where there is evidence of strong associations there are many patients who do not carry the HL-A type in question. However, in the studies dealing with chronic hepatitis, multiple sclerosis, psoriasis, and ankylosing spondylitis very striking HL-A associations have been observed. The most likely explanation for this dilemma probably derives from the fact that susceptibility or resistance to disease is controlled by multiple genes including HL-A genes. If such is the case then expression of a disease state could reflect linkage disequilibrium between particular HL-A genes and genes closely linked to HL-A and consequently HL-A antigens would only serve, in some instances, as markers for the genes that are actually involved. Whatever the case may be the development of methods for identifying specific immune responses in humans will be of prime importance in the establishment of these associations. Toward this end we have been studying a large control population with respect to

* Supported in part by grants: NCI-N01-CB-43853X, NIH-PHS 1R01 HDO8145-01, NIH-PHS 2P01-HL06314-13, and NIH-PHS HE-06314.

cell-mediated immunity in vivo by measuring delayed-type skin reactions to selected antigens; and in vitro, by the blastogenic response of lymphocytes to the same antigens. The marked variation in response to one of these antigens, streptokinase-streptodornase (SK/SD),[1] prompted us to study this phenomenon in detail. In this study we provide evidence for the association of HL-A 5 with immune responsiveness in vitro to streptococcal antigens.

Materials and Methods

Test Lymphocytes. Peripheral lymphocytes were isolated and purified (24) from 217 randomly selected individuals ranging in age from 25 to 65 yr. These individuals were judged to have normal immune function by the following criteria: capacity to stimulate and respond in mixed leukocyte cultures; normal response to phytohemagglutinin, concanavalin A, and pokeweed mitogen; normal immunoglobulin levels; normal proportion of peripheral blood B cells as determined by immunofluorescent staining of cell surface immunoglobulins and by complement receptors; and a normal range of "T" cells as indicated by T-cell rosettes and a T-cell cytotoxicity assay.

HL-A Serotyping. HL-A antigens were determined with a standard two-stage dye exclusion microcytotoxicity test (25) using a panel of 115 well-characterized antisera capable of detecting all defined HL-A and most W specificities.

Streptococcal Antigens. The antigen preparation employed in this study (SK/SD) was obtained from American Cyanamid Co., Lederle Laboratories Div., Pearl River, N. Y. Although this type of preparation is rather crude, it is one of the recommended antigens (26) commonly used as a skin test for the assessment of pre-existing immunity. In order to minimize heterogeneity we obtained a large quantity of antigen preparation distributed in 2-ml ampules from a single lot. The contents of each ampule were dissolved in 2 ml sterile distilled water assayed for protein (27) and kept at 4°C. Although this stock solution was stable at 4°C for approximately 2 mo, extensive testing necessitated preparing of new stock solution on a weekly basis. Dilutions were made each day in culture medium immediately before use. Streptococcal nucleases were isolated by the method of Marker and Gray (28).

Assay System. 10^5 purified lymphocytes were incubated in triplicate in a CO_2 environment at 37°C in Microtest II (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) culture plates. Each well contained 0.2 ml of Eagle's minimal essential medium with addition of penicillin, streptomycin, L-glutamine, pooled human serum (20% vol/vol), and varying concentrations of SK/SD. The same pool of human serum and employed throughout this study. After 120 h, 50 μl of tritiated thymidine ([^{3}H]TdR) solution (1 μCi/ml) was added to each well. The plates were incubated an additional 24 h, harvested, and assayed for radioactivity.

Results

Test lymphocytes incubated in vitro with varying concentrations of streptococcal antigens showed three types of response: (*a*) response over the entire range of antigen concentrations; (*b*) low response to only the highest antigen concentrations; and (*c*) no response to any antigen concentration. The data is presented in Fig. 1 in which the rate of incorporation of [^{3}H]TdR is plotted as a function of antigen concentration. It can be seen that lymphocytes from individuals defined as responders (curve A) are reactive over a concentration range from 10 to 6,000 ng SK/SD/ml. The low responder (curve B) reactivity decreases rapidly with decreasing antigen concentration reaching a minimum at 100 ng/ml, while the nonresponders (curve C) do not react at any concentration of antigen. The frequency of HL-A antigens in the various population segments is shown in Table I. Statistical analysis of these distributions by chi-square (Table II) reveals that

[1] *Abbreviations used in this paper:* [^{3}H]TdR, tritiated thymidine; SK/SD, streptokinase-streptodornase.

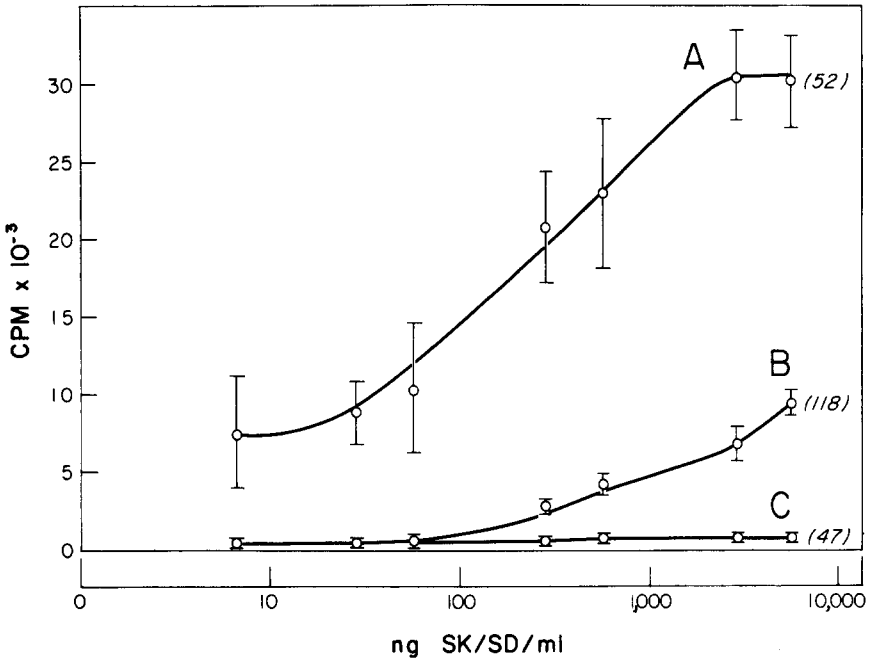

FIG. 1. In vitro response to streptococcal antigens. Triplicate cultures of 10^5 purified lymphocytes were incubated in a CO_2 environment at 37°C. After 120 h 50 μl of [^{3}H]TdR (1 μCi/ml) was added to each culture. The cultures were incubated an additional 24 h, harvested, and assayed for radioactivity. Curves A, B, and C represent responders, low responders, and nonresponders, respectively. Numbers in parentheses indicate the sample size and the vertical bars represent the standard error of the mean.

the low and nonresponder populations have the same HL-A antigen frequency whereas the incidence of HL-A 5 is significantly increased in the responder population when compared to low responders alone (P = 0.0003) or to the combined low and nonresponder population (P = 0.0001).

The use of antigens such as SK/SD for the assessment of cell-mediated immunity is predicated on the fact that the healthy adult population, having had prior immunologic experience with streptococcal antigens will react both in vivo with a delayed-type skin reaction and in vitro by lymphocyte blastogenesis. Since SK/SD is a mixture of proteins, it was surprising to find a relatively large number of individuals that failed to respond in vitro. Furthermore, the striking association between responsiveness and HL-A 5 might be expected from an antigen of restricted heterogeneity rather than the heterogeneous preparation used in this study. One possible explanation for the observed response pattern might be that SK/SD, upon dilution, manifests only one effective stimulatory component while at higher concentrations a more heterogeneous reactivity is elicited. While definitive characterization of SK/SD stimulation will have to await fractionation and purification, it has been established that SK/SD contains only one nuclease, i.e., nuclease A. This fact and the availability of purified samples of streptococcal nucleases A, B, C, and D allowed us to study the blastogenic effects of these enzymes on the reactivity of lymphocytes from

TABLE I

Frequency of HL-A Antigens in Responders and Nonresponders to Streptococcal Antigens

First locus					Second locus				
Antigen	Responders*	Low responders‡	Non-responders§	Total‖	Antigen	Responders*	Low responders‡	Non-responders§	Total‖
	%	%	%	%		%	%	%	%
HL-A 1	30.8	28.8	19.2	27.4	HL-A 5	30.8	5.9	8.5	12.5
HL-A 2	48.0	59.2	53.2	55.2	HL-A 7	13.4	28.8	23.8	25.8
HL-A 3	23.0	28.0	34.0	28.1	HL-A 8	15.4	23.7	21.3	21.2
HL-A 9	25.0	13.6	19.2	17.5	HL-A 12	21.1	32.2	34.0	30.0
HL-A 10	9.6	14.4	12.8	12.9	HL-A 13	5.8	8.5	4.3	6.9
HL-A 11	5.8	11.0	12.8	10.2	W5	21.1	14.4	3.4	14.3
W19	23.0	17.8	12.8	18.0	W10	13.4	14.4	23.4	18.2
W28	9.6	6.8	6.4	7.4	W14	9.6	4.2	4.3	5.5
					W15	28.8	12.7	14.8	17.0
					W16	1.9	6.8	14.8	7.4
					W17	3.8	4.2	2.1	5.0
					W18	5.8	6.8	2.1	5.5
					W22	0.0	1.7	0.0	0.92
					W27	1.9	6.8	4.3	5.0

* *n*, 52.
‡ *n*, 118.
§ *n*, 47.
‖ *n*, 217.

TABLE II

Chi-square Analysis of HL-A Antigen Frequency Distribution

Antigen	A vs. B	*P*	A vs. C	*P*	A vs. B + C	*P*	B vs. C
HL-A 1	0.0664		1.7660		0.4428		1.6293
HL-A 2	1.8513		0.2583		1.4432		0.5171
HL-A 3	0.4432		1.4634		0.8574		0.5974
HL-A 9	0.3390		0.4889		2.6549		0.8168
HL-A 10	0.7354		0.2481		0.6578		0.0754
HL-A 11	1.1658		1.4623		1.4328		0.1009
W19	0.6433		1.7643		1.2087		0.6215
W28	0.4109		0.3472		0.5033		0.0085
HL-A 5	19.0321	0.00001 (0.0003)	7.5870	0.01	21.0841	0.000004 (0.0001)	0.3591
HL-A 7	4.6482	0.05	4.8637	0.05	5.4432	0.025	0.1548
HL-A 8	1.5055		0.5761		1.3836		0.1140
HL-A 12	2.1480		2.0675		2.5241		0.0516
HL-A 13	0.3740		0.1179		0.1389		0.8872
W5	1.1943		4.4361	0.05	2.6345		2.0315
W10	0.0265		1.6405		0.3597		1.9311
W14	1.8856		1.0794		2.1849		0.0082
W15	6.4652	0.025	2.7805		6.7275	0.01	0.1384
W16	1.6979		5.5914	0.025	2.9744		2.6776
W17	0.0139		0.2481		0.0049		0.4269
W18	0.0608		0.8443		0.0074		1.4105
W22	0.8918		—		0.6361		0.8063
W27	1.6979		0.4569		1.4065		0.3762

A, B, and C refer to responder, low responders and nonresponders, respectively. p values for all chi-squares above 4.000 are given. Numbers in parentheses refer to P values corrected for the number of HL-A antigens. Corrected P values less than 0.05 are given. Corrections were made using the following expression: $P = 1 - (1 - p)^{22}$, where p is the uncorrected value and the exponent relates to the number of HL-A antigens studied.

responders and nonresponders to SK/SD. It can be seen in Table III that lymphocytes from responders to SK/SD (II) have a significantly higher reactivity to concentrations of nucleases A and D in the threshold range than do lymphocytes from nonresponders (I). In the case of nucleases B and C the blastogenic reactivity is considerably lower; the threshold level for nuclease B is 10-fold higher than A, C, and D; and there is no apparent difference between SK/SD responders (II) and nonresponders (I). These data not only suggest that the observed response pattern to SK/SD is mediated by nuclease A (streptodornase) but support the idea that responsiveness is genetically controlled. In an effort to rule out possible residual streptococcal infection as a cause of the variation in response, serum antistreptolysin-O titers were measured in all individuals studied. No statistical difference in titer was observed between responders, low responders, and nonresponders. However, the possibility still remains that antibodies against components of SK/SD, present only in responder lymphocytes, mediate the observed blastogenesis. In this context it is of interest to point out that McCarty (29) found considerable variation in antibody response to nuclease A in scarlet fever patients. In a study of 90 patients, 34 showed

TABLE III

Blastogenic Response (cpm) to Streptococcal Nucleases in the Threshold Concentration Range

HL-A phenotype	Nuclease A (0.09 U/ml)	Nuclease B (0.9 U/ml)	Nuclease C (0.09 U/ml)	Nuclease D (0.09 U/ml)
I*				
3, 7, 10	4,400	1,784	1,087	6,422
1, 2, 8, 10	7,947	2,442	841	6,073
9, W19, 7, W17	735	249	0	217
1, 3, 7	4,169	0	0	49
1, 2, W17, W18	9,452	1,766	741	4,324
	$\bar{X}$ = 5,341	$\bar{X}$ = 1,248	$\bar{X}$ = 889	$\bar{X}$ = 3,417
II‡				
2, 9, W5	68,222	4,959	3,791	33,169
1, 11, 5	32,165	1,272	497	27,217
2, 3, 5, 7	41,989	898	0	16,344
2, 9, 5, 12	20,841	2,116	0	9,759
2, W19, 5, W14	12,918	200	569	2,806
	$\bar{X}$ = 35,227	$\bar{X}$ = 1,889	$\bar{X}$ = 1,619	$\bar{X}$ = 17,859
I vs. II				
t	3.07	0.7	1.22	2.52
P	<0.01	NS	NS	<0.025

* Nonresponders to SK/SD.

‡ Responders to SK/SD.

significant antibody titers to nuclease A, 33 did not have measureable amounts of this antibody during the course of the study, and the remaining individuals had low titers, once again suggestive of a possible genetic control.

Discussion

The apparent association of HL-A 5 with responsiveness in vitro to streptococcal antigens is interesting for two reasons: (*a*) it may represent the first demonstration of an in vitro probe with which to study immune response genes in general, and (*b*) more specifically it may provide an in vitro model for studying immunogenetic aspects of streptococcal disease.

Although the precise mechanism by which specific histocompatibility antigens influence the susceptibility or resistance to disease is not known, Snell (30) has suggested the following possibilities: the histocompatibility antigen of the cell surface might be a receptor site for the attachment of an infectious agent; an infectious agent might share antigenic determinants with host histocompatibility antigens whereby the host would be tolerant to these antigens; and finally that histocompatibility antigens themselves are not involved directly but the genes controlling susceptibility are linked to genes coding for histocompatibility antigens. In the first two situations susceptibility would be dominant in the heterozygote whereas in the third case resistance would be dominant in the heterozygote.

Of particular relevance to streptococcal disease is the observation that heat-killed group A Streptococci have the ability to induce a state of altered reactivity to skin homografts in guinea pigs (31) which may come about as a result of shared antigens or alterations of the animal's antigens by the injected material. Efforts to relate this observation to problems in humans revealed that the M protein from beta hemolytic group A Streptococcus pyogenes type 1 completely inhibited the cytotoxic antibody activity of antisera to HL-A 1, 2, 3, 7, 8, and 9 whereas analogous M proteins from types 3, 4, 5, 6, 12, and 14 had little or no inhibitory activity (32). The authors interpreted this data to mean that M1 protein has a structure common to human histocompatibility antigens. In a more recent study (33) M1 protein was found to stimulate human peripheral blood lymphocytes in culture to undergo a dose-dependent increase in DNA synthesis. However, the degree of stimulation was not related to the serum anti-M1 titer of the lymphocyte donor, the effectiveness of M1 antigen in blocking the cytotoxic activity of HL-A antisera, or to the HL-A phenotype. The lack of agreement in these studies with respect to inhibition of cytotoxic HL-A antibody activity makes one question the interpretation that M1 protein has significant structural similarities to human histocompatibility antigens. Furthermore, the lack of any HL-A association probably derives from an insufficient sample size (n = 9).

Since streptococci appear to contain many substances capable of stimulating blastogenesis in lymphocytes, it is quite possible that in some cases clear-cut associations between HL-A antigens and immune responsiveness will be established while in other cases no association will be apparent. On the other hand, the lack of sufficient characterization of stimulatory material may provide an element of confusion, whereby lymphocyte blastogenesis may be induced by only one streptococcal component present as a contaminant of other preparations (34). This notion seems unlikely, however, in view of the marked difference in reactivity observed in lymphocytes from responders and nonresponders to different streptococcal nucleases (Table III). The selective nature of the nuclease-mediated blastogenesis in this study and the variation in antinuclease A antibody levels observed by McCarty (29) in scarlet fever patients, if related, provide a rational basis for postulating the existence of a specific genetic control mechanism governing immune responsiveness to streptococcal antigens which appears to be linked to the major histocompatibility system. Recently, in vivo studies from several laboratories have provided suggestive evidence for the existence of HL-A-linked immune response genes (35–38). If histocompatibility-linked immune response genes are important in determining susceptibility or resistance to disease in general, then a search for associations will depend not only upon the demonstration that specific immune response genes are indeed linked to the major histocompatibility loci but also on the availability of techniques for determining their phenotypic expression in man. The observed association of HL-A 5 to in vitro blast transformation by streptococcal antigens is another step forward in this direction.

Summary

Lymphocytes, from randomly selected individuals having normal immune function, when incubated in vitro with varying concentrations of streptococcal antigens, responded in three ways: (*a*) response over the entire antigen concentration range, i.e., responders; (*b*) low response to only the highest antigen concentrations; and (*c*) no response at any antigen concentration. Frequency

distribution analysis of these groups indicated that a significant association occurred between the ability to respond and HL-A 5.

Received for publication 7 October 1974.

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