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Ernest D. Gray

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Research Article

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The geometric means of antibody levels against streptolysin O, nuclease B, and nicotinamide adenine dinucleotidase showed no consistent differences between the control group and the group of RHD subjects. Group A streptococci were isolated from the tonsils of ~25% of both groups of subjects.

The RHD patients clearly had a depressed cellular immune response to the two purified streptococcal extracellular antigens. The equal frequency in recovery of group A streptococci from tonsils and the absence of consistent difference in titers of humoral antibodies to streptococcal extracellular antigens, particularly nuclease B, suggest that this differential response is not due to a lower level of stimulation by repeated exposure to group [...]

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Cellular Immune Responses to Extracellular Streptococcal Products in Rheumatic Heart Disease

ERNEST D. GRAY, *Departments of Pediatrics and Biochemistry, Medical School, University of Minnesota, Minneapolis, Minnesota 55455*

LEWIS W. WANNAMAKER, *Departments of Pediatrics and Microbiology, Medical School, University of Minnesota, Minneapolis, Minnesota 55455*

ELIA M. AYOUB, *Department of Pediatrics, University of Florida College of Medicine, Gainesville, Florida 32610*

AZIZ EL KHOLY, *Biomedical Research Center for Infectious Diseases, Cairo, Egypt*

ZAHIRA H. ABDIN, *Rheumatic Heart Center, Cairo University, Cairo, Egypt*

ABSTRACT The lymphocyte transformation responses to purified preparations of two extracellular products of group A streptococci (blastogen A and nuclease B), to phytohemagglutinin, and to *Candida albicans* antigen were measured in tonsillar and peripheral blood lymphocytes from patients with rheumatic heart disease (RHD) and suitably matched nonrheumatic (control) subjects.

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due to a lower level of stimulation by repeated exposure to group A streptococcal products.

INTRODUCTION

The development of acute rheumatic fever (ARF)¹ and rheumatic heart disease (RHD) is generally held to involve some reaction of the immune system to group A streptococci or their products. The development of antibodies crossreacting with streptococcal cellular constituents and with components of cardiac cells (1), as well as alterations in various aspects of the cellular immune response (2-8) is, at least, presumptive evidence for immune mechanisms in the pathogenesis of rheumatic heart injury.

The changes in cellular immune function are diverse and intriguingly complex. Leukocyte migration inhibition in response to streptococcal membranes is increased in ARF and may persist for at least 5 yr after the initial attack (7). The one-way mixed lymphocyte culture response of ARF cells to allogeneic lymphocytes may be depressed (8). Several reports indicate altered lymphocyte transformation response to streptococcal antigens in poststreptococcal disease. Using either whole organisms or streptococcal substances in various stages of impurity, a depressed lymphoproliferative response has generally been observed in ARF and in RHD (2-6). However, other workers have reported an elevated blastogenic response in ARF and RHD (9, 10). The complexity of the streptococcal preparations used in these various studies makes it difficult

Dr. L. W. Wannamaker is a Career Investigator of the American Heart Association.

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¹Abbreviations used in this paper: ARF, acute rheumatic fever; PHA, phytohemagglutinin; RHD, rheumatic heart disease.

to interpret the results. Studies with purified substances might resolve some of these discrepancies.

The development of ARF and RHD is apparently a consequence of group A streptococcal infections of the upper respiratory tract; infections with group A streptococci at other sites, e.g., skin, do not lead to these complications (11). The role of the tonsils in this process is unknown, but it is possible that local factors could be important in the pathogenetic process leading to heart damage. For example, tonsillar lymphocytes may be a different subpopulation(s) and/or may respond to streptococcal antigens in a different manner than lymphocytes of the peripheral blood (12). In earlier studies of the blastogenic response of tonsillar lymphocytes to impure preparations of streptococcal products, conflicting findings of their reactivity have been reported (13, 14).

In this investigation the responses of tonsillar and peripheral blood lymphocytes from RHD patients were compared with those from nonrheumatic patients. Homogeneous preparations of two streptococcal extracellular products were used: streptococcal blastogen A (15), a product recently shown to have mitogenic properties, and streptococcal nuclease B,² an antigen that specifically stimulates previously sensitized lymphocytes. Phytohemagglutinin (PHA) and a preparation of *Candida albicans* antigen were used respectively, as nonspecific mitogen and specific antigen controls. The response of tonsillar and peripheral blood lymphocytes to both streptococcal antigens was markedly depressed in subjects with RHD as compared to a control group. The alteration more likely was not a consequence of differential exposure of the subjects to streptococcal antigens.

METHODS

Antigens. Streptococcal blastogen A and streptococcal nuclease B were purified from the crude extracellular products of group A streptococcal strain C203S³ as previously described (15). The proteins were homogeneous as indicated by sodium dodecyl sulfate gel electrophoresis. *C. albicans* antigen was a crude preparation obtained from Hollister-Stier Laboratories, Spokane, Wash. PHA was obtained from Difco Laboratories, Detroit, Mich.

Antibody determinations. The level of serum antibody against streptolysin O was determined by the method of Edwards (16). Antistreptococcal nuclease B (anti-DNase B) was measured by the microtechnique of Nelson et al. (17). Antistreptococcal nicotinamide adenine dinucleotidase (anti-NADase) was determined by the method of Ayoub and Ferretti (18).

Bacteriological analysis. Swabs from the external surface and interior of left and right tonsils were streaked onto sheep

blood agar plates. After overnight incubation at 37°C, the streptococcal colonies were categorized as α - or β -hemolytic and colonies exhibiting β -hemolysis were isolated and serologically grouped (19).

Lymphocyte transformation assay. Blastogenic stimulation was measured by [³H]thymidine incorporation by a gradient-separated monocyte population from peripheral blood and tonsils. The tonsils were minced with scissors, forced through a stainless steel screen and resuspended in Eagles' minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) containing 100 U/ml of penicillin, 100 μ g/ml streptomycin, 50 μ g/ml of gentamycin, and 2 mmol/ml L-glutamine (MEM-PSG). The cell suspension was fractionated by centrifugation on a Ficoll-Hypaque gradient as previously described (15). The samples of blood were diluted with an equal volume of MEM-PSG and the monocyte population isolated in the same manner. Triplicate 100- μ l samples of each antigen dilution (in MEM-PSG-5% fetal calf serum), were transferred to wells of standard tissue culture microtiter plates containing 10⁵/100 μ l lymphocytes. The PHA plates were incubated for 3 d, and the microbial preparations for 5 d at 37°C in an atmosphere containing 5% CO₂. At these times, 0.5 μ Ci of [methyl-³H]thymidine was added and the plates incubated further for 18 h. Controls without antigen or PHA were included in each run. The cells were harvested and washed on glass fiber paper using a semiautomatic cell harvester. The radioactivity was measured in a toluene-based cocktail by scintillation counting.

Population study. Tonsillar tissue and samples of peripheral blood were obtained with the informed consent of individuals residing in Cairo, Egypt, who were scheduled for tonsillectomy on the advice of their physicians. The diagnosis of RHD was confirmed by history and careful physical examination. In patients with RHD, tonsillectomy was performed an average of 2.3 yr (1–5 yr) after the last clinically diagnosed attack of rheumatic fever. None of the rheumatic patients was in heart failure or showed evidence of rheumatic activity. None was taking medication other than penicillin. None of the rheumatic or nonrheumatic patients exhibited signs of frank nutritional deficiency. 23 control subjects were matched by age and socioeconomic status with 20 RHD patients. A detailed history and physical examination were carried out on control subjects to exclude a past history suggestive of rheumatic fever and evidence of subclinical RHD. The average ages of the RHD group and the control group were 18.1 and 16.0 yr, respectively. The study was carried out during annual visits to Cairo over a period of 3 yr. The results for each of the years were similar.

RESULTS

Lymphocyte blastogenic response. The mean response of peripheral blood lymphocytes to PHA in the group of RHD patients was indistinguishable from that of the control subjects (Fig. 1). The tonsillar lymphocyte response was also very similar in both groups (Fig. 2) although the dose-response relationships were markedly different from those of the peripheral blood lymphocytes. The proliferation of the tonsil-derived cells was inhibited to a significantly greater extent by higher levels of the lectin. The similarity in response to this nonspecific T cell mitogen suggests that the proportion of lymphocytes capable of a proliferative response was similar in the RHD and control groups.

² Gray, E. D., A. E. Flores, and S. C. Marker. Submitted for publication.

³ Gray, E. D., S. C. Marker, and A. E. Flores. Submitted for publication.

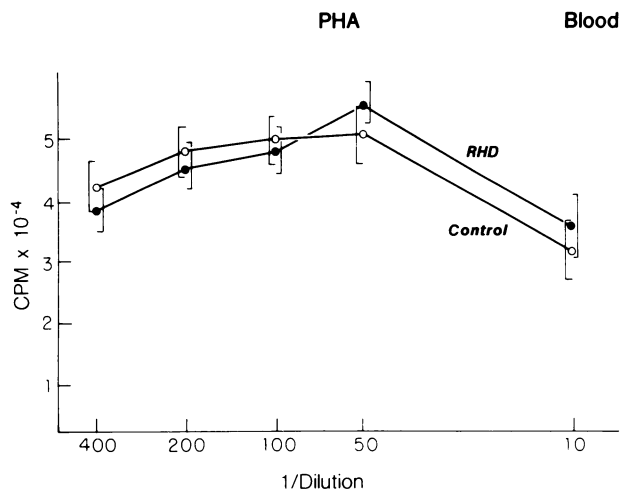


FIGURE 1 Blastogenic response of peripheral blood lymphocytes to PHA. Lymphocyte stimulation by various concentrations of PHA was measured as described in Methods. The results were corrected for the thymidine incorporation by non-stimulated cells and averaged. The bars indicate SEM. ●, RHD subjects; ○, control subjects.

The blastogenic response of lymphocytes to the streptococcal extracellular antigens was different in the two groups of patients. Streptococcal blastogen A stimulated the response of lymphocytes from control subjects to a greater extent than those from the RHD group (Figs. 3 and 4). This lesser response was observed in peripheral blood and tonsillar lymphocytes from RHD subjects. The dose-response curves were similar in both groups, with the RHD group at a lower level. The results obtained with tonsil-derived lymphocytes, in particular, indicate that this lower response

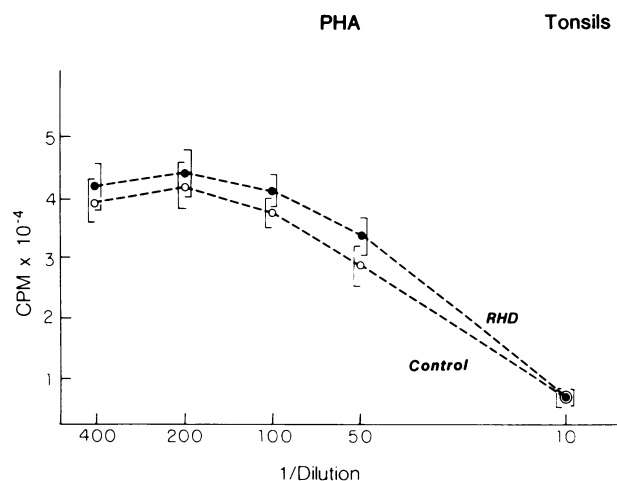


FIGURE 2 Blastogenic response of tonsillar lymphocytes to PHA. Lymphocyte stimulation was measured and results reported as described in Fig. 1. ●, RHD subjects; ○, control subjects.

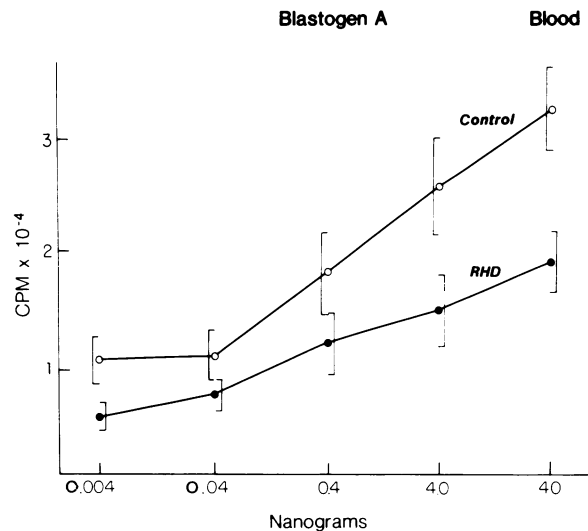


FIGURE 3 Blastogenic response of peripheral blood lymphocytes to streptococcal blastogen A. Lymphocyte stimulation was measured and results reported as described in Fig. 1. ●, RHD subjects; ○, control subjects.

would not be overcome with increased amounts of blastogen A since similar levels of thymidine incorporation were obtained at both of the higher doses. The RHD lymphocytes are apparently refractory to the streptococcal blastogen as compared with those of the control group.

A similar result was observed when lymphocytes were challenged with streptococcal nuclease B (Figs.

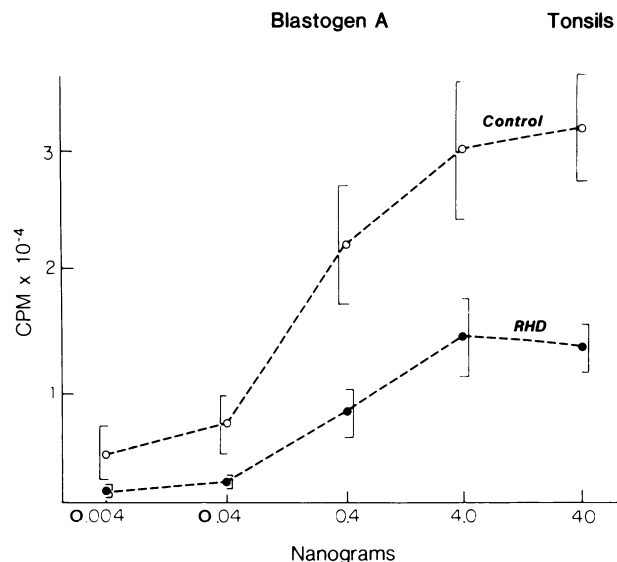


FIGURE 4 Blastogenic response of tonsillar lymphocytes to streptococcal blastogen A. Lymphocyte stimulation was measured and results reported as described in Fig. 1. ●, RHD subjects; ○, control subjects.

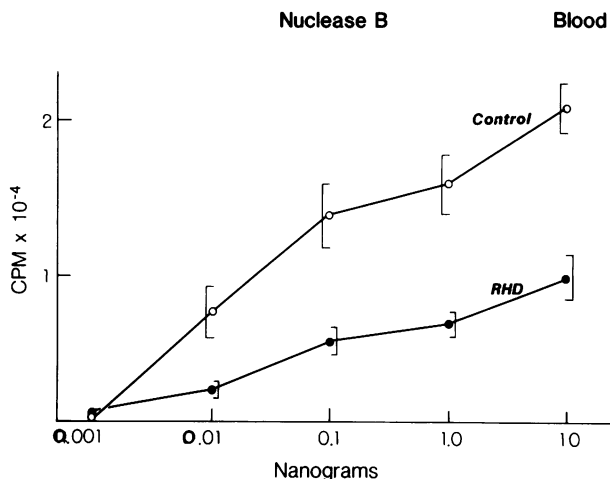


FIGURE 5 Blastogenic response of peripheral blood lymphocytes to streptococcal nuclease B. Lymphocyte stimulation was measured and results reported as described in Fig. 1. ●, RHD subjects; ○, control subjects.

5 and 6). This antigen stimulated proliferation of peripheral blood and tonsillar lymphocytes to a markedly greater extent in the control group than in the RHD subjects. The level of response to nuclease B is lower than that with blastogen A even in the control group, but clearly, with this antigen also, fewer lymphocytes from RHD subjects were stimulated to proliferate.

Although considerable variation was observed in individual results, analysis of the dose-response data indicated that the differences in response between RHD and control subjects were in most cases highly significant (Table I). The *P* values of differences in blastogen A stimulation were lower in the tonsillar

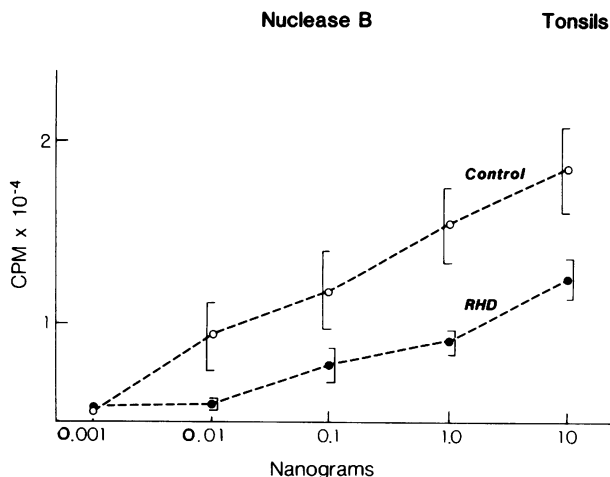


FIGURE 6 Blastogenic response of tonsillar lymphocytes to streptococcal nuclease B. Lymphocyte stimulation was measured and results reported as described in Fig. 1. ●, RHD subjects; ○, control subjects.

lymphocytes than in those from peripheral blood. The opposite was observed with nuclease B. Although with this antigen the levels of response were in all instances lower than those obtained with blastogen A, the variations in individual results were less, and the *P* values smaller.

C. albicans was selected as a nonstreptococcal antigen, to which all subjects might have been exposed, and thus might serve to define whether the differential response in RHD subjects was confined to streptococcal products. Unfortunately the *Candida* antigen under the conditions employed resulted in no measurable stimulation of lymphocyte proliferation (results not shown). The amounts of incorporated radioactivity were indistinguishable from those of cells incubated with no added antigen. The same results were obtained in each year of the study using new lots of antigen.

Streptococcal antibody levels. The lowered lymphocyte transformation response in RHD subjects could be a reflection of a difference in recent exposure (sensitization) to streptococcal antigens, or a generally reduced capability of the immune system to respond to streptococcal antigens. As an approach to these questions, the levels of serum antibodies to nuclease B, streptolysin O, and NADase were measured in RHD and nonrheumatic subjects (Table II). The mean titers of antibodies against nuclease B and NADase were essentially similar in both subject groups. A somewhat lower level of antistreptolysin O was present in the RHD group. The level of these antibodies does not appear to be consistently altered in the patients with RHD as compared with controls. Of particular interest is the finding that the mean antibody titer for nuclease B was at least as high in the rheumatic as in the non-rheumatic group, whereas the cellular immune responses to this antigen were quite different in the two patient groups. In rheumatic and control individuals, the titer of antinuclease B showed no correlation with the lymphoproliferative response to nuclease B.

Streptococcal flora of the tonsils. Swabs taken from the exterior and interior of the tonsils from RHD subjects and controls were cultured for the presence of streptococci (Table III). The majority of tonsils from both groups contained β -hemolytic streptococci representing at least five Lancefield groups. In some instances multiple groups or types were isolated from the same patient. Group A strains were recovered from the tonsils of 4 of the 18 patients with RHD (22%) and 5 of the 19 nonrheumatic patients (26%). There was no significant difference in the streptococci isolated from the exterior or interior of the tonsils.

DISCUSSION

The lymphocyte blastogenic response to two homogeneous streptococcal extracellular products was signifi-

TABLE I
*Statistical Significance of Differences in Lymphocyte Blastogenic Response between RHD and Control Groups**

Nanograms	Blastogen A†				
	40	4	0.4	0.04	0.004
P blood	2×10^{-3}	0.02	0.09	0.15	0.04
P tonsils	2×10^{-4}	9.8×10^{-5}	4.5×10^{-3}	0.018	0.17
Nanograms	Nuclease B‡				
	100	10	1	0.1	0.01
P blood	7.8×10^{-6}	3×10^{-5}	4×10^{-4}	2.6×10^{-3}	0.74
P tonsils	9.5×10^{-3}	1.1×10^{-3}	0.036	0.017	0.85

* Data were analyzed using Student's *t* test and the null hypothesis.

† The peripheral blood data represent 23 RHD subjects and 20 controls; the tonsillar data represent 19 RHD subjects and 14 controls.

§ The peripheral blood data represent 23 RHD subjects and 20 controls; the tonsillar data represent 16 RHD subjects and 14 controls.

cantly lowered in patients with rheumatic heart disease as compared with nonrheumatic subjects. The reactivity to PHA, a polyclonal T cell mitogen, was however, no different in RHD and control subjects. The differential response in RHD subjects is evidently not due to a general decrease in T cell responsiveness but may be specifically associated with reaction to streptococcal antigens. A nonstreptococcal antigen, *C. albicans*, stimulated little or no blastogenic response in the assay system used; thus it is not established whether the lowered response is associated only with streptococcal antigens. However, the results are consonant with previous reports of a relatively depressed response in peripheral blood lymphocyte transformation to streptococcal antigens in patients with ARF or RHD (2-6).

In contrast to the suppressed cellular immune response in patients with RHD reported here, Read et al. (7) described an exaggerated cellular reactivity in patients with rheumatic fever which lasted for at least 5 yr after the initial attack. Several factors may account for the differences in these two studies. The technique of migration inhibition of peripheral blood leukocytes was used by Read et al. (7), whereas we used lymphocyte incorporation of tritiated thymidine as a measure of cellular response. Moreover, these workers examined responses to particulate components of the

streptococcal cell (cell walls and membranes), whereas we studied responses to soluble extracellular products that are apparently not found in significant amounts in association with streptococcal cellular constituents. Because only particulate antigens worked in their system, Read et al. did not examine purified soluble antigens (cellular or extracellular).

In interpreting our results it was considered that a lower response would be expected in RHD subjects who might be protected from exposure to streptococcal antigens by antimicrobial prophylaxis. This possibility is not supported by the indirect evidence available: (a) the levels of antistreptococcal antibodies in the patients with RHD are not greatly or regularly different from the control group; (b) similar frequencies and distributions of streptococci were found in the tonsils of RHD and control subjects; (c) patient interviews indicated that many were not consistent in the main-

TABLE III
Streptococci Isolated from Tonsillar Tissue

	RHD		Control	
	n*	%	n*	%
α-hemolytic	14	(78)	11	(58)
β-hemolytic	15	(83)	13	(68)
Group				
A	4		5	
B	1		1	
C	4		3	
F	3		0	
G	2		5	

* n represents the number of patients from whom the various organisms were isolated. A total of 18 RHD and 19 control subjects were studied.

TABLE II
*Antistreptococcal Antibody Levels**

	RHD	Controls
Antistreptolysin O	130 (107-158)	202 (176-231)
Antinuclease B	254 (224-288)	236 (203-275)
Anti-NADase	137 (109-172)	171 (133-220)

* Geometric mean titers with SEM in parentheses.

tenance of prophylaxis. As judged by these findings, the exposure of the RHD group to streptococci was not significantly different and presumably their immune systems had been presented with a streptococcal challenge generally comparable to that of controls.

The previously reported observations of lowered responsiveness to streptococcal antigens could have been due to a dose-response phenomenon, but the present results obviate this possibility. In the studies with lymphocytes from patients with ARF (2, 3, 5, 6) the results might have included an artifactual lowering of lymphoproliferation by the effect of aspirin (20) or steroids (21), but this was not a factor in the responses of the RHD patients reported here, none of whom were receiving or had recently received these drugs. These findings suggest that the lymphocytes from RHD subjects are relatively refractory to stimulation by streptococcal antigens, or are different in the kinetics of their proliferative response.

With the mitogens and antigens used in the present study, tonsillar and peripheral blood lymphocytes reacted in a similar manner. This confirms and extends the results of Drucker et al. (14) using less purified preparations. The similarity in response suggests that the tonsillar lymphocytes are subject to the same influences that modulate the response of peripheral blood lymphocytes. It has been reported that tonsillar lymphocytes, in some patients, exist in a preactivated state with an elevated level of spontaneous lymphoproliferation (14). In such patients, the blastogenic response to mitogen (PHA) or antigen is depressed in both tonsillar and peripheral blood lymphocytes. This phenomenon was not observed in the present study; although the unstimulated response was somewhat higher in tonsillar than peripheral blood lymphocytes there was no decrease in the stimulated tonsillar response.

A suggested mechanism for the lowered response to streptococcal antigens in RHD subjects was that the responding lymphocytes in the blood of control subjects are somehow sequestered in sites where they interact with antigens (4). Tonsils can be such a reservoir of streptococcal antigens but in fact, in the present study hemolytic streptococci were recovered with approximately equal frequency from the tonsils of both groups, and the response of the tonsillar lymphocytes to streptococcal antigens in the RHD subjects was low compared to that in the peripheral blood. Certainly, if lymphocytes are sequestered in tonsillar tissue, those isolated are not as responsive as those from control subjects.

Crude culture filtrates of group A streptococci have been shown to stimulate the proliferation of T lymphocytes (22). Purified streptococcal blastogen A also stimulates blastogenesis in T cell populations although interaction with non-T cells appears necessary for maxi-

mal reactivity.⁴ The streptococcal products used in the present study represent one protein (blastogen A) with relatively nonspecific lymphocyte stimulating properties and one (nuclease B) which would specifically stimulate transformation of primed lymphocytes. The results clearly indicate that the lowered response characteristic of patients with RHD occurs with both proteins. Since blastogen A acts as a polyclonal T cell stimulator, this suggests that the depression in response is not limited to specifically primed lymphocytes. Some degree of specificity is, however, indicated by the identity of response of the RHD and control subjects to PHA. It is possible that the responsiveness in RHD is affected by a deficiency in a responding subpopulation. Williams et al. (23) have reported an increased proportion of T cells forming "active rosettes" in patients with ARF but did not report on patients with inactive RHD.

Another mechanism could involve a suppressor cell interaction. The blastogenic response to a crude preparation of extracellular products from group C streptococci as well as to partially purified preparations of group A extracellular nucleases is associated with an increased frequency of human histocompatibility antigen (HLA) B5 (24). In mice, the immune response genes are closely linked to HLA loci, and the formation of specific suppressor cells is involved in the immune response gene controlled response to a specific antigen (25). If the HLA association of response to streptococcal antigens in man actually involves immune response genes, these might be related to the generation of suppressor cells resulting in the lowered response observed in RHD subjects.

The understanding of the mechanism of altered cellular immune response clearly requires further investigation that may ultimately lead to insights into the pathogenesis of RHD.

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