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Compartmentalization of Cells Bearing "Rheumatic" Cell Surface Antigens in Peripheral Blood and Tonsils in Rheumatic Heart Disease

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Monoclonal antibodies that recognize "rheumatic" antigens of peripheral blood non-T cells were used to study the compartmentalization of such cells in peripheral blood and tonsils of individuals with rheumatic heart disease (RHD) and suitable control subjects. The peripheral blood of most (71%) of the 42 individuals with RHD contained cells reacting with monoclonal antibody 83S19.23 or 256S.10, whereas these cells were present in only 17% of the 41 control subjects ($P < .02$). However, none of 21 individuals with RHD had such cells in their tonsils, although they were present in the tonsils of 50% of the 40 control subjects ($P < .03$). These results may reflect a failure in RHD of organ-specific homing of cells with the epitopes recognized by the antibodies. The presence of these cells in tonsils may be important in the immune response to streptococcal pharyngeal infection, and their absence in RHD may be involved in the unusual immune responses characteristic of this disease.

Elements of the cell surfaces of some peripheral blood mononuclear cells from individuals with a history of rheumatic fever (RF) appear to be antigenically distinct from those of the majority of the population [1]. This difference has been defined with human alloantisera that react with non-T cells (those not possessing a receptor for sheep red blood cells [SRBCs]) of >75% of individuals who have had documented RF. Monoclonal antibodies with similar specificities have been developed that also allow the definition of "rheumatic" antigens on non-T cells [2, 3] in a similar proportion of individuals having had RF. Previous studies of the distribution of these antigens have examined their presence on the mononuclear cells of peripheral blood. The present study was directed at their distribution in both peripheral blood and tonsils in individuals having inactive rheumatic heart disease (RHD) compared with those having recurrent or chronic tonsillitis.

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Informed consent was obtained from the individuals undergoing tonsillectomy, who donated peripheral blood and tonsillar tissue.

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Subjects and Methods

The individuals with RHD and control subjects were residents of Cairo. RHD was diagnosed by careful history and physical examination. None of the individuals with RHD had heart failure or showed signs of rheumatic activity (i.e., fever or elevated erythrocyte sedimentation rate) within a year of the study, and none showed obvious signs of nutritional deficiency.

Detailed history and physical examination of the control subjects excluded past or present RF or RHD. The average ± SD age of those with RHD was 11.4 ± 2.5 years and of the control subjects, 9.8 ± 4.8 years (difference not significant). Samples of peripheral blood and tonsillar tissue were obtained from individuals undergoing tonsillectomy on the advice of their physician. The indication cited for tonsillectomy was recurrent or chronic tonsillitis. The subjects were afebrile and had normal erythrocyte sedimentation rates. The individuals with RHD had

approximately two incidents of pharyngitis during the year before study, whereas the control subjects had six incidents. Each tonsil was cultured and examined for the presence of group A *Streptococcus*. The recovery of this organism was infrequent and not different in the RHD and control groups. This finding was consistent with the attending physicians' reports that all of the volunteers had taken penicillin or sulfonamides for at least one month before tonsillectomy. Some of the individuals with RHD either had already undergone tonsillectomy or were not scheduled for this operation, so only blood was studied from this group.

Mononuclear cells were isolated from blood and tonsils by gradient centrifugation. Mononuclear cells were rosetted with SRBCs, and T cells were separated from non-T cells by gradient centrifugation as previously described [4, 5]. The blood was diluted with an equal volume of Eagle's MEM, layered on Ficoll-Paque® (Pharmacia, Uppsala, Sweden), and centrifuged at 650 *g* for 30 min. The mononuclear cells were removed from the gradient interface and washed twice with MEM, and a portion was subjected to rosette depletion for removal of T lymphocytes. The tonsils were minced with scissors and shaken for 1 min with MEM. Mononuclear cells were isolated from the resulting cell suspension by the same method used with blood. T cell depletion was accomplished by SRBC rosetting. The mononuclear cells from blood and tonsils were resuspended in MEM containing 20% fetal calf serum (previously adsorbed with SRBCs) and then incubated for 5 min at 37 C with an equal volume of 5% SRBCs in 20% adsorbed fetal calf serum. After incubation the cell suspension was layered on Ficoll-Paque and centrifuged as described above. The mononuclear cells were removed, washed twice, and used for cell-marking studies. These nonrosetting cells are referred to as non-T cells. The non-T population isolated by these methods included 50% B lymphocytes, 30% monocytes, and <3% residual T cells. This distribution was not determined for each preparation.

The monoclonal antibodies used in the study were 83S19.23 and 256S.10. They were produced in response to immunization of mice with non-T cells from peripheral blood of individuals with a history of RF and were prepared in the Pharmaceuticals Research Department, Ciba-Geigy, Ltd. (Basel, Switzerland). These antibodies have specificity similar to that of the human alloantisera previously shown to react with the B cells of individuals with a history of RF [1, 2].

The study was conducted in two annual visits to Cairo. In one year the cell surfaces were stained by an indirect immunofluorescence technique, and in the other year an immunoperoxidase technique was used.

Indirect immunofluorescence staining was done by a method similar to that described by Abramson et al. [6]. For each non-T sample of tonsil and blood, three aliquots were stained: monoclonal antibodies 83S19.23 and 256S.10 and a control for determination of nonspecific adsorption. The slides were carefully labeled with a code and read in a blinded man-

Table 1. Immunofluorescence assay of "rheumatic" cells.

Tissue, group, antibody reactivity	Monoclonal antibody 83S19.23	Monoclonal antibody 256S.10	Nonspecific binding
Blood			
RHD			
Positive	2.7 ± 0.44 (13)	2.1 ± 0.54 (4)	1.3 ± 0.25 (25)
Negative	1.4 ± 0.24 (12)	0.8 ± 0.21 (21)	
Control			
Positive	4.9 ± 2.2 (4)	. . . (0)	0.9 ± 0.21 (20)
Negative	0.6 ± 0.17 (16)	0.6 ± 0.20 (20)	
Tonsils			
RHD			
Positive	. . . (0)	. . . (0)	0 (6)
Negative	0.26 ± 0.11 (6)	0.08 ± 0.08 (6)	
Control			
Positive	2.5 ± 0.5 (4)	2.6 ± 0.57 (3)	1.1 ± 0.65 (18)
Negative	0.5 ± 0.3 (14)	1.0 ± 0.49 (15)	

NOTE. Data are mean ± SE percentages of positive cells (no. of individuals).

Table 2. Immunoperoxidase assay of "rheumatic" cells.

Tissue, group, antibody reactivity	Monoclonal antibody 83S19.23	Monoclonal antibody 256S.10	Nonspecific binding
Blood			
RHD			
Positive	5.9 ± 0.65 (15)	5.2 ± 0.8 (7)	1.9 ± 0.3 (17)
Negative	2.4 ± 0.1 (2)	2.6 ± 0.3 (10)	
Control			
Positive	9.3 ± 0.25 (2)	10.3 (1)	2.1 ± 0.2 (21)
Negative	1.5 ± 0.2 (19)	1.4 ± 0.14 (20)	
Tonsils			
RHD			
Positive	. . . (0)	. . . (0)	1.3 ± 0.32 (11)
Negative	1.1 ± 0.3 (11)	1.3 ± 0.26 (11)	
Control			
Positive	5.7 ± 2.5 (3)	6.7 ± 2.6 (7)	1.4 ± 0.26 (22)
Negative	1.7 ± 0.35 (19)	1.9 ± 0.34 (15)	

NOTE. Data are mean ± SE percentages of positive cells (no. of individuals).

ner. An individual was regarded as "positive" for a given monoclonal antibody if the proportion of cells binding the monoclonal antibody was significantly ($P < .05$ by χ^2 analysis) greater than the proportion of cells binding the serum control. Sufficient numbers of cells were counted so that the probability (β) of not detecting a difference of ≥1% was <.10 (range of cells counted, 200–1,800).

A summary of the results obtained with the immunofluorescence method is shown in table 1. The data represent mean values of the staining results and illustrate the background staining obtained with this method and the differences between positive and negative groups. The technique detects the binding of monoclonal antibodies to only a small fraction of non-T cells, but the group of positive individuals clearly have a higher proportion of these cells than do the negative groups, which are indistinguishable from the nonspecific serum control. The percentages of labeled cells in the positive RHD groups are not significantly different from those in the positive control groups.

The immunofluorescence method requires that slides be read soon after preparation, and so that this limitation on the study of increased numbers of samples was overcome, an immunoperoxidase staining method was developed. Immunoperoxidase staining was accomplished by the avidin-biotin complex method with a kit from Vector Laboratories (Burlingame, Calif) [7, 8]. The non-T cells were spread on carefully labeled glass slides, and a circle ~2 cm in diameter was inscribed around the cells, which were allowed to dry overnight. The slides were immersed in 95% ethanol for 5 min, allowed to dry, and then immersed in PBS (pH 7.4) for 10 min. The slides were blotted dry around the circles containing the cells, 30 µl of diluted horse serum was added to the circles, and the slides were incubated at room temperature (~23 C) in a humid chamber for 30 min so that nonspecific binding of immunoglobulin was blocked. They were then immersed in PBS for 10 min and blotted, and 30 µl of diluted monoclonal antibody was added. After incubation for 30 min the slides were washed again in PBS and blotted, and 30 µl of biotin-labeled antiserum to mouse immunoglobulin was added. After incubation for 30 min and a wash in PBS, 30 µl of biotin-avidin-peroxidase solution was added and incubated for 30 min. After a PBS wash and blotting, 50 µl of diaminobenzidine–hydrogen peroxide substrate was added, and the slides were incubated for 10 min. They were then washed in distilled water and dried. Control slides were similarly stained without monoclonal antibody. The dried slides were transported to the University of Minnesota Medical School (Minneapolis) and read in a blinded and coded manner. Positive cells were those with black granules around a greater part of their periphery. A slide was considered positive when it had a significantly greater number of positive cells than did the horse serum control slide, as determined by χ^2 analysis.

Table 2 summarizes the immunoperoxidase results and illustrates the higher proportion of monoclonal antibody–binding cells in positive individuals, as

Table 3. Cell surface labeling of peripheral blood and tonsillar non-T cells.

Tissue, monoclonal antibody	Antibody positivity	
	RHD	Control
Blood		
83S19.23	67 (28/42)*	17 (7/41)
256S.10	26 (11/42)*	5 (2/41)
83S19.23 or 256S.10	71 (30/42)*	17 (7/41)
83S19.23 and 256S.10	21 (9/42)*	5 (2/41)
Tonsils		
83S19.23	0 (0/21)†‡	20 (8/40)
256S.10	0 (0/21)†‡	38 (15/40)§
83S19.23 or 256S.10	0 (0/21)†‡	50 (20/40)§
83S19.23 and 256S.10	0 (0/21)‡	8 (3/40)

NOTE. Data are percentages (no. of subjects positive/no. tested).

* Significantly different from control subjects ($P < .02$).

† Significantly different from control subjects ($P < .03$).

‡ Significantly different from blood from individuals with RHD ($P < .02$).

§ Significantly different from blood from control subjects ($P < .002$).

compared with the immunofluorescence method. With the immunoperoxidase method the proportion of labeled cells in individuals considered negative is not significantly different than the proportion in slides with nonspecific serum instead of monoclonal antibody.

Although the percentage of reactive cells detected by the two methods differed, the proportion of individuals with or without these cells was not significantly different in the two years of the study. Therefore the data on individuals from the two years were pooled for analysis.

Results

Cell surface labeling of peripheral blood non-T cells. Most individuals with RHD had peripheral blood non-T cells, with antigenic structures identified by the monoclonal antibodies 83S19.23 and 256S.10 (table 3). A significantly lower proportion of the control subjects had non-T cells bearing these epitopes in their peripheral blood ($P < .02$). In both the RHD and control groups, most of the positive individuals had cells reacting with antibody 83S19.23, and fewer had cells reacting with antibody 256S.10. Cells marked by this latter antibody were most frequently present in individuals with cells also marked by antibody 83S19.23. These antibodies evidently are to a cell surface structure commonly present on the peripheral blood mononuclear cells of individuals with RHD.

Cell surface labeling of tonsillar non-T cells. In remarkable contrast to the results with peripheral blood, the non-T cells from tonsils of individuals with RHD were not labeled by antibody 83S19.23 or 256S.10 (table 3). None of the 21 subjects with RHD possessed tonsillar non-T cells with cell surfaces identified by these antibodies. However, the tonsils of 50% of the control subjects contained cells reactive with these antibodies. Furthermore, most of the positive subjects possessed cells marked by antibody 256S.10 rather than antibody 83S19.23, as seen in peripheral blood. Clearly, in both the RHD and control groups, the populations of non-T cells present in tonsils are different than those in peripheral blood.

Relation between blood and tonsils in individuals. Both blood and tonsils were studied from 17 individuals with RHD and from 34 individuals without RHD. This approach permitted a comparison between the blood and tonsils of each of these individuals for the presence or absence of a significant proportion of monoclonal antibody–labeled non-T cells. An individual's blood or tonsil was considered "positive" if it contained a significantly greater proportion of non-T cells binding either or both monoclonal antibodies than did their serum controls. Each of these individuals could therefore have positive tonsillar non-T cells, positive blood non-T cells, or positive non-T cells in both compartments or in neither compartment. Table 4 shows the unexpected and highly significant ($P < .001$) difference between the RHD group and the control group in the distribution of positive non-T cells between blood and tonsils. Individuals whose blood was positive for the "rheumatic" antigens identified by these monoclonal antibodies were much more likely to be in the RHD group ($P < .005$, φ coefficient = 0.39; table 5). How-

Table 4. Distribution of non-T cells bearing "rheumatic" antigens in blood and tonsils.

Study group	Blood + tonsils −	Blood + tonsils +	Blood − tonsils −	Blood − tonsils +	Total
RHD	14	0	3	0	17
Control	3	1	19	11	34

NOTE. Data are no. of subjects. These results are a summary of those obtained from individuals whose blood and tonsils were both studied. The difference between the RHD and control groups was significant ($\chi^2 = 28.2$, $P < .001$).

Table 5. Analysis of association of "rheumatic" antigen with RHD.

Study group	Blood*		Tonsil†		Blood and tonsils‡	
	Positive	Negative	Positive	Negative	Blood + tonsils −	Blood − tonsils +
RHD	14	3	0	17	14	0
Control	4	30	12	22	3	11
Total	18	33	12	39	17	11

NOTE. Data are no. of subjects.
* χ^2 = 24.7, $P < .001$; φ coefficient for RHD if blood was positive = 0.70.
† χ^2 = 7.8, P = .005; φ coefficient for RHD if tonsils were negative = 0.39.
‡ χ^2 = 18.1, $P < .001$; φ coefficient for RHD if blood was positive and tonsils were negative = 0.80.

ever, those individuals whose blood non-T cells bore the "rheumatic" antigens and whose tonsillar non-T cells did not were the most likely to be in the RHD group. Those individuals whose tonsillar cells were positive and whose blood did not contain positive cells were most likely to be in the group without RHD ($P < .001$, φ coefficient = 0.80; table 5).

Discussion

The presence of particular antigens on the surface of non-T cells in individuals with RF or RHD has been observed in populations as disparate as those in India, the United States, and Egypt [3, 9]. These cell surface structures are characteristically found in most individuals with this form of disease after streptococcal infection. Their molecular characteristics and function are not well defined, but as they may represent expression of Ia-like genes [1], they might play a role in the altered immune responses associated with RF and RHD [4, 5, 10]. The present study does not permit conclusions regarding the functional role of these non-T cells bearing "rheumatic" antigens. However, other studies have shown that non-T cells are a requirement for a T cell lymphoproliferative response to some group A streptococcal products [5]. Moreover, subjects with RHD from a similar population have an altered function of blood and tonsillar non-T cells that results in lower T cell proliferation. These individuals also have lower relative rates of T suppressor/cytotoxic cell (T8) proliferation and higher T4/T8 cell ratios after stimulation of their tonsillar cells by streptococcal blastogen A [11]. The relation between these altered immune responses and the "rheumatic" non-T cells may be involved in the pathogenesis of RHD.

In the population studied here, the presence of non-T cells bearing the "rheumatic" antigens in the blood and their absence in the same individual's tonsils were most strongly associated with RHD. In contrast, the absence of non-T cells bearing the "rheumatic" antigens in the blood and their presence in the same individual's tonsils were most strongly associated with an absence of RHD. These relations were observed even though the control group experienced more pharyngitis than did the RHD group. Because both groups showed no acute signs of inflammation, it seems likely that the differences in compartmentalization were not due to acute changes in the tissue distribution of non-T cells. Possibly the differences were due to an underlying genetic difference alone or in combination with environmental factors. The current study groups were selected because both groups had ample time (mean age, 10 years) and exposure that made repeated pharyngitis due to group A streptococci very likely. This situation was important for identification of the association of potential immunocyte markers with RHD because streptococcal pharyngeal infections are necessary for the expression of a propensity to RHD. The quantitation of RHD risk associated with these monoclonal antibody markers will be best defined by a prospective study from infancy in populations at high risk for RHD.

One of the most important and apparently absolute determinants for the development of RF and RHD after group A streptococcal infection is that the infection take place in the upper respiratory tract [12]. Among the local factors that must be considered to explain this observation is the role of the tonsils and related pharyngeal lymphoid tissue. It is possible that the tonsils may be the focus of immunologic aberrations that lead to the development of disease after streptococcal infection. However, the composition of the freshly isolated mononuclear cell populations with respect to B cells, total T cells, or helper and

suppressor T cell subsets is not significantly altered in the tonsils of individuals with RHD [11].

The homing of intestine-associated B lymphocytes to the mammary glands during pregnancy is well documented [13–15]. This homing is most likely dependent on B cell surface characteristics, with those B lymphocytes bearing antibody specific for antigens delivered to the maternal intestine and emigrating to the breast. It is possible that individuals are put at increased risk for the development of RF and RHD if appropriate non-T cells have not homed to the pharyngeal lymphoid tissue before or during episodes of group A streptococcal pharyngitis.

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