Clin. exp. Immunol. (1970) **6**, 573–586.

IMPAIRED LYMPHOCYTE STIMULATION BY SOME STREPTOCOCCAL ANTIGENS IN PATIENTS WITH RECURRENT APHTHOUS STOMATITIS AND RHEUMATIC HEART DISEASE*

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(*Received* 4 *August* 1969)

SUMMARY

The effects of pathogenic and non-pathogenic streptococci, streptococcal cell wall products, and phytohaemagglutinin on human peripheral leucocyte cultures from four groups were studied. These groups were: (1) normals, (2) patients with aphthous stomatitis, (3) patients with Behçet's disease, and (4) patients with rheumatic heart disease. The degree of lymphocyte stimulation by these materials was measured by uptake of [^{3}H]thymidine into DNA *in vitro*. In normals, patients with aphthous stomatitis, and Behçet's disease, the human pathogenic group A streptococci produced significantly greater stimulation of DNA synthesis than did the less pathogenic non-haemolytic streptococci. Lymphocytes from patients with aphthous stomatitis showed significantly less stimulation of DNA synthesis than comparable normal controls when exposed to heat-killed Streptococcal 2A, organisms which have been implicated in the disease. Human pathogenic strains of group A streptococci which have been implicated in rheumatic heart disease stimulated significantly less *in vitro* proliferation of lymphocytes from patients with rheumatic heart disease than of those from a comparable group of normal controls. This hypo-responsiveness persisted when the patients' lymphocytes were cultured in normal human serum. The chronically ill Behçet's patients' lymphocytes did not differ significantly from normal. These observations indicate a deficiency of the cellular response of certain patients to antigens from organisms thought to be aetiologically related to their disease.

INTRODUCTION

To the practising clinician, recurrent aphthous stomatitis presents a difficult diagnostic and therapeutic challenge. It must be differentiated from oral ulcers of viral aetiology

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* Presented in part at the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, 1969.

(Graykowski *et al.*, 1966) and may be associated with a muco-cutaneous-occular syndrome described as Behçet's disease (Curth, 1946; Lehner, 1967a).

Although the aetiology of aphthous stomatitis remains obscure, the most recent hypothesis by Lehner implicates an autoimmune mechanism since he has demonstrated haemagglutinating antibodies to foetal oral mucosa cell extracts (Lehner, 1964) and increased *in vitro* stimulation of lymphocytes (Lehner, 1967b) by saline extracts of oral mucosa in these patients. It is also of interest that immunosuppressive drugs such as cyclophosphamide have produced striking clinical improvement in several patients with aphthous stomatitis (Francis, unpublished data).

In contrast, Graykowski *et al.* (1966) have obtained pure cultures of *Streptococcus sanguis* (prototype strain $2A_{2+3}$HOT) from mutiple isolations to recurrent aphthous lesions. They have also shown these patients to have greater than normal delayed type skin reactivity to intradermal skin challenge with a vaccine prepared from such heat-killed organisms. They have therefore suggested that recurrent aphthous stomatitis may represent a specific streptococcal hypersensitivity.

The technique of lymphocyte transformation can be used to investigate the degree and specificity of an *in vivo* immune reaction *in vitro*. It has been shown that normal human lymphocytes respond to a variety of streptococcal antigens *in vitro* (Hirschhorn *et al.*, 1964; Francis, Oppenheim & Barile, 1969; Pachman & Fox, 1968). We have therefore studied the *in vitro* effect of *Str. sanguis* strain 2A, other heat-killed virulent and non-virulent streptococci, their cell-wall extracts, and an M protein preparation on peripheral blood leucocyte cultures from the following groups: (1) normal controls, (2) patients with recurrent aphthous stomatitis, (3) patients with Behçet's disease whose recurrent oral lesions closely resemble those of recurrent aphthous stomatitis, and (4) patients with severe rheumatic heart disease and a previous history of multiple streptococcal infections which served as a positive control since their streptococcal hypersensitivity state is much more well established than in aphthous stomatitis.

MATERIALS AND METHODS

Human leucocytes (WBC) cultures

Human peripheral leucocytes were obtained from: (1) Thirty normal adult volunteers (mean age 35·6 years). (2) Thirteen patients at the N.I.H. Clinical Center with documented recurrent aphthous stomatitis (mean age 34·3 years). (3) Seven patients with Behçet's disease (mean age 36 years) with oral lesions that were similar to those in recurrent aphthous stomatitis. Patients in this group were also chronically ill with multiple systemic involvement and were on varying doses of steroid therapy. (4) Fourteen patients with inactive rheumatic heart disease (mean age 41·3 years), selected on the basis of a past history of severe streptococcal illness, that were kindly made available for study by the National Heart Institute prior to their cardiac repair or extensive diagnostic procedures. These patients were frequently receiving prophylactic Digoxin and penicillin. None of them was receiving aspirin. Their anti-streptolysin-O titres were within normal limits (166 Todd units). None of the patients was in acute distress or extremely debilitated. Whenever possible the patients' cell cultures were set up simultaneously with that of a normal volunteer of matched age, sex and race.

Sixty to 100 ml of blood was drawn into a heparinized syringe and mixed with 6%

dextran in saline (Abbott Laboratories) at a volume of 25% of the initial blood volume drawn. This mixture was transferred to tubes and placed at a 30° angle at 37°C for $1\frac{1}{2}$ hr to allow sedimentation of the red blood cells. The supernatant WBC rich plasma was removed. The WBCs were resuspended in Eagle's medium (MEM, containing 50 units penicillin and 100 μg streptomycin/ml) and 20% autologous plasma at a final concentration of $1{\cdot}5 \times 10^6$ WBC/ml. On occasion, following sedimentation with dextran in saline, the cells were centrifuged at 500 g for 10 min at 26°C and the supernatant plasma saved. The cells were resuspended and washed twice with cold Earle's balanced salt solution (pH 7·2) containing 10% decomplemented foetal bovine serum. The cells were then resuspended at a concentration of $1{\cdot}5 \times 10^6$ WBC/ml in MEM containing either 20% of the patient's, or normal human plasma. Three-millilitre aliquots of this WBC suspension were transferred to $\frac{1}{4}$-oz. sterile French square bottles. One-fourth of a millilitre of saline was added to control cultures and streptococcal antigens, as indicated, were added to replicate cultures. Cultures were done in duplicate or triplicate. The cells were incubated in tightly-capped bottles for 5 days at 37°C. Four and one-half hours before harvesting, two microcuries of tritiated thymidine (TdR^3H specific activity 6·7 Ci/mM, New England Nuclear, Boston, Massachusetts) were added to each culture. Cultures were then processed to determine their total acid precipitable TdR^3H uptake in a Packard scintillation counter (Oppenheim & Perry, 1965). The counts per minute were corrected for quenching and expressed in terms of absolute efficiency in disintegrations per minute (dpm) per culture.

Cell morphology and differential counts

Smears of cell cultures were prepared, air dried, fixed with methanol, and stained by the Jenner–Giemsa method. The cell morphology and percent blasts, lymphocytes and macrophages present in the representative sets of cultures were determined using previously described criteria (Oppenheim & Perry, 1965).

Culture additives

(1) *Phytohaemagglutinin* (PHA, obtained from G. Hitchings, Burroughs-Wellcome).

(2) *Smallpox vaccine* (calf lymph type-72P12, Wyeth Laboratories, Marietta, Pennsylvania) used the equivalent of one-third the skin test dose per culture.

(3) *Heat-killed streptococci*: the following streptococcal strains were used as stimulants at a final concentration of 1×10^7 organisms/ml of cell suspension which represented previously determined optimal concentrations for culture stimulation:

Streptococcus pyogenes group A type 12 (55 × 193), type 14 (59 × 171), and type 24 (60 × 203) (kindly provided by Dr R. Cole, N.I.H. Bethesda, Maryland); *Str. sanguis* strain $2A_{2+3}$HOT; *Str. sanguis* strain 14A Bi 1–3 (isolated and characterized in this laboratory); Eli Lilly strain *Str. indifferent*; *Str. fecalis* group D (isolated and characterized in this laboratory); and *Str.* sp. group C (ATCC No. 12960—animal origin). Although this latter group of non-virulent strains under the proper circumstances can cause minor infections in man, they are less pathogenic than the above Lancefield group A strains.

(4) *Streptococcal products*: Streptolysin S (SLS, obtained from A. Bernheimer, New York University) 50 haemolytic units/culture; Streptolysin O (SLO, Bacto-Difco, Detroit, Michigan) 0·1 ml of 1 : 25 dilution/culture.

(5) *Cell extracts*: 0·05 mg culture; purified M protein prepared from Streptococcal group A type 12 (obtained from E. Fox, University of Chicago) 0·003 mg culture; acid

cell-wall extract (CHO) as per Lancefield (see below) prepared from above *Str. sanguis* strain 2A and Streptococcal group A type 12, 0·3 ml of a 1 : 10 dilution leucocyte culture.

Preparation of the streptococcal antigens

The organisms were grown in Todd–Hewitt broth and incubated at 37°C for 18–24 hr. The medium was supplemented with 5–10% heat inactivated foetal bovine serum for growth of the streptococci. The viable bacterial count was determined by a dilution plate count method using sheep blood agar. The organisms were concentrated by centrifugation for 20 min at 16,300 ***g*** in a refrigerated Sorvall model RC2–B centrifuge. The cells were resuspended and washed twice with sterile saline and were killed by heating at 60°C for 1 hr. Sterility was determined by inoculating aliquots of the bacterial suspension onto blood agar and into Todd–Hewitt broth. The final stock suspensions of the organisms were made in sterile saline at a concentration of 1×10^8 colony forming units/ml and hereafter referred to as organisms/ml.

Preparation of the cell-wall extract (CHO)

A modification of the Lancefield acid extraction method (Lancefield, 1928) was used. The streptococci were grown, harvested and counted as described above. Streptococci obtained from 400 ml of Todd–Hewitt broth (10^8–10^9 organisms/ml) were concentrated by centrifugation, resuspended in 4 ml of 0·2 N HCl and placed in a boiling water bath for 10 min with constant shaking. The suspension was sedimented by centrifugation at 12,000 ***g*** and the supernatant collected. Four drops of phenol red indicator were added to the supernatant and the pH was adjusted to 7·0 with 1·0 N NaOH. The precipitate was removed by centrifugation at 12,000 ***g*** and the supernatant containing the CHO was diluted 1 : 10 with saline. Sterility testing was performed as described above. This method of Lancefield has been found to yield fractions of both group and type specific antigens including M protein. The protein and carbohydrate content of these CHO preparations were determined by the Lowry (Lowry *et al.*, 1951) and Anthrone (Scott & Melvin, 1953) techniques respectively and were found to contain, in the case of Streptococcus 2A 0·14 mg/ml protein + 0·91 mg/ml carbohydrate, and from Streptococcal strain type 12 0·21 mg/ml protein + 0·87 mg/ml carbohydrate. Pilot studies of the dose response curve of the lymphocytes of normal subjects with these CHO preparations revealed the optimal stimulating dose to be 1·4 μg protein (9·1 μg carbohydrate) for Streptococcal strain 2A and 2·1 μg protein (8·7 μg carbohydrate) for Streptococcal strain type 12 per culture, and these concentrations were used subsequently in the study. The lyophilized preparation of M protein from Streptococcal strain type 12 was reconstituted with MEM to a concentration of 0·01 mg/ml.

Statistical analysis

The disintegrations per minute of TdR^3H incorporated by proliferating lymphocytes were distributed in such a way that logarithmic transformation of the geometric means of replicate cultures yield normally distributed data. The comparison of log transformed geometric means from simultaneously tested patient groups and controls was accomplished on paired comparisons using a Student's *t*-test. Intra-group comparisons of paired stimulated and unstimulated responses were done in a similar fashion.

RESULTS

The response of normal subjects' lymphocytes to antigens from pathogenic and non-pathogenic streptococci are shown in Table 1. It is evident that the human pathogenic streptococci, i.e. *Str.* group A types 12, 14 and 24 were all better stimulants than the non-pathogenic *Str. sanguis* strain 2A, 14A, *Str. indifferent*, and those representatives of the other Lancefield groups C and D. It should be noted that the *Str.* 2A cell-wall extract (CHO) did not stimulate leucocytes significantly more than the whole-killed *Str.* 2A organism. However, the *Str.* type 12 cell-wall extract (CHO) was a significantly more effective stimulant than the whole-killed *Str.* type 12. It should be noted here that PHA was used as a control stimulant in every set of patient and normal cell cultures.

TABLE 1. Normal subjects' *in vitro* lymphocyte response

Stimulant	No.	Geometric mean dpm	Multiple increase over saline	Student's *t*-test	Multiple increase over *Str.* 2A	Student's *t*-test	Multiple increase over *Str.* T12 organism	Student's *t*-test
1. Saline control	30	3,784						
2. PHA	29	166,474	44.0	0·001				
3. SLO	17	54,487	14·4	0·001				
4. SLS	13	36,915	9.7	0·001				
5. *Str.* 2A organism	22	18,328	4·8	0·001				
6. *Str.* 2A CHO	27	19,169	5·1	0·001	1·0	NS		
7. *Str.* type 12 organism	23	46,544	12·3	0·001	2·5	0·001		
8. *Str.* type 12 CHO	24	75,863	20·0	0·001	4·1	0·001	1·6	0·05
9. *Str.* type 12 M prot.	21	22,839	6.0	0·001	1·2	NS	0·49	NS
10. *Str.* type 14 organism	7	35,150	9·3	0·005	1·9	NS	0·76	NS
11. *Str.* type 24 organism	12	41,976	11·1	0·001	2·3	0·05	0·9	NS
12. *Str.* indiff. organism	12	9,677	2·5	0·01				
13. *Str.* gp. D organism	7	4,095	1·1	NS				
14. *Str.* 14A organism	4	2,880	0·76	NS				
15. *Str.* gp. C organism	6	7,334	1·9	0·005				

NS, Not significant.

Table 2 shows that the aphthous patients' *in vitro* leucocyte response to these stimuli was similar to that of the normal group in most respects. Their response to human pathogenic streptococci was, again, much greater than the response to the non-pathogenic streptococci. As in the normal group, the response to the cell-wall extract (CHO) from the group A *Str.* type 12 organisms was significantly greater than to the whole-killed organism. However, in contrast to the normal controls, the aphthous patients' lymphocyte stimulation by the *Str.* 2A (CHO) was significantly greater than by the whole *Str.* 2A organism.

Fig. 1 compares the geometric mean disintegrations per minute (dpm) of the normal subjects *versus* the patients with aphthous stomatitis for each individual stimulant. A Student's *t*-test was used to test for any significant differences between the normal and patient populations (see Table 5). The histogram represents the geometric mean dpm per

culture for each stimulant as indicated. Data on the *Str.* group C and D organisms and *Str. sanguis* strain 14A Bi 1–3 has been omitted here to simplify the chart and because the response to these organisms was similar to the degree of stimulation as seen by *Str. sanguis* strain 2A and *Str.* Eli Lilly strain indifferent. Of all the comparisons seen in Fig. 1, only the response to *Str.* 2A organisms was significantly reduced in patients with aphthous stomatitis in comparison with the normal subjects ($P = 0{\cdot}025$). The differences observed in Fig. 1 between the normals and aphthous patients for the type 14 and type 24 organisms were not statistically significant. An analysis of variance of the type 14, type 24, and 2A organism data in Fig. 1 shows no statistically significant differences between the variances for each pair of data, thus making the Student's *t*-test applicable.

TABLE 2. Aphthous patients' *in vitro* lymphocyte response

Stimulant	No.	Geometric mean dpm	Multiple increase over saline	Student's *t*-test	Multiple increase over *Str.* 2A	Student's *t*-test	Multiple increase over *Str.* T12 organism	Student's *t*-test
1. Saline control	13	4,609						
2. PHA	13	184,385	40·0	0·001				
3. SLO	6	64,188	13·9	0·005				
4. SLS	3*	46,476	10·1	—				
5. *Str.* 2A organism	10	8,678	1·9	0·01				
6. *Str.* 2A CHO	12	26,597	5·8	0·001	3·1	0·001		
7. *Str.* type 12 organism	11	52,003	11·3	0·001	6·0	0·001		
8. *Str.* type 12 CHO	11	81,341	17·6	0·001	9·4	0·001	1·6	0·01
9. *Str.* type 12 M prot.	10	30,472	6·6	0·005	3·5	0·001	0·59	NS
10. *Str.* type 14 organism	4	17,746	3·9	NS				
11. *Str.* type 24 organism	4	87,186	18·9	NS				
12. *Str.* indiff. organism	3*	4,865	1·1	—				
13. *Str.* gp. D organism	2*	22,600	4·9	—				
14. *Str.* 14A organism	2*	1,059	0·23	—				
15. †								

NS, Not significant.

* Insufficient sample size.

† *Str.* Gp. C—not done.

Table 3 represents the *in vitro* leucocyte response in a group of six patients with Behçet's disease. Although this sample size was not sufficiently large to warrant as precise a statistical analysis, their lymphocyte responses closely resembled that of the normal group.

The *in vitro* leucocyte response to the various streptococcal stimuli in a group of selected patients with severe rheumatic heart disease (RHD) with documented histories of severe streptococcal involvement is shown in Table 4. In contrast with the normal response, these patients' lymphocytes did not respond significantly more to the group A type 12 and 24 organisms than to the *Str.* 2A organism. There was no significant increase in stimulation by the *Str.* type 12 CHO over that of the *Str.* type 12 organism. The *Str.* type 14 and 24 also show significantly lower degree of lymphocyte stimulation than the *Str.* type 12.

Fig. 2 compares the geometric mean dpm of normals and patients with rheumatic heart disease. There appears to be a general depression of response in the rheumatic heart group to all the streptococcal stimulants as compared to the response in normals. Those comparisons of significance are summarized in Table 5. It should be noted here that SLO and SLS stimulated normal and aphthous patient cells markedly; however, in the rheumatic heart group this effect was depressed. This observation is of interest since SLO is a specific stimulant (Hirschhorn *et al.*, 1964) while SLS is not (Stollerman & Bernheimer, 1950;

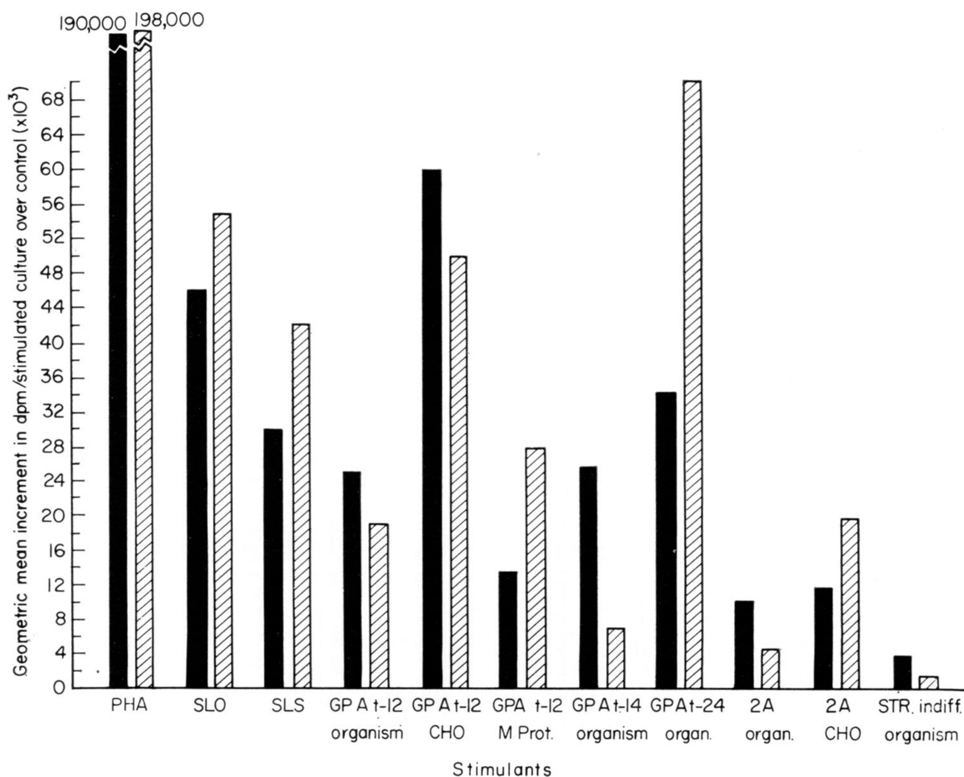

FIG. 1. Illustrates the comparison of the *in vitro* leucocyte culture response to the various stimuli between normals (solid columns) and patients with aphthous stomatitis (hatched columns). The only comparison significantly different is with the strain 2A organism ($P < 0{\cdot}025$).

Humphrey, 1949) and does, in fact, behave as a non-specific mitogen for peripheral blood lymphocytes (Naspitz & Richter, 1968; Taranta, Cuppari & Quagliata, 1969). Also included in Table 5 is data from smallpox stimulated leucocyte cultures from four rheumatic heart patients and five normals. Although this data is obtained from 6-day cultures rather than 5-day cultures (as for streptococcal antigens), it shows no difference in response between the rheumatic heart patients and normals.

Morphological analysis on representative cultures throughout all of these experiments revealed that the degree of lymphocyte transformation observed generally corresponded to the degree of tritiated thymidine uptake by replicate cultures.

TABLE 3. Behçet patients' *in vitro* lymphocyte response

Stimulant	No.	Geometric mean dpm	Multiple increase over saline	Student's *t*-test	Multiple increase over *Str.* 2A	Student's *t*-test	Multiple increase over *Str.* T12 organism	Student's *t*-test
1. Saline control	7	2,631						
2. PHA	6	170,959	65·0	0·001				
3. SLO	6	66,105	25·1	0·005				
4. SLS	6	8,656	3·3	NS				
5. *Str.* 2A organism	6	12,754	4·8	NS				
6. *Str.* 2A CHO	6	16,859	6·4	0·025	1·3	NS		
7. *Str.* type 12 organism	6	33,780	12·8	0·01	2·6	NS		
8. *Str.* type 12 CHO	5	61,953	23·5	0·005	4·9	NS	1·8	NS
9. *Str.* type 12 M prot.	4	11,234	4·3	NS	0·88	NS	0·33	NS
10. *Str.* type 14 organism	3*	15,970	6·1	—				
11. *Str.* type 24 organism	6	24,484	9·3	0·005				
12. *Str.* indiff. organism	5	6,007	2·3	NS				
13. *Str.* gp. D organism	3*	3,337	1·3	—				
14. *Str.* 14A organism	2*	3,968	1·5	—				
15. *Str.* gp. C organism	3*	6,530	2·5	—				

NS, Not significant.
* Insufficient sample size.

TABLE 4. Rheumatic heart disease patients' *in vitro* lymphocyte response

Stimulant	No.	Geometric mean dpm	Multiple increase over saline	Student's *t*-test	Multiple increase over *Str.* 2A	Student's *t*-test	Multiple increase over *Str.* T12 organism	Student's *t*-test
1. Saline control	14	3,258						
2. PHA	14	269,085	82·6	0·001				
3. SLO	8	21,846	6·7	0·01				
4. SLS	8	11,914	3·7	NS				
5. *Str.* 2A organism	12	9,822	3·0	0·005				
6. *Str.* 2A CHO	8	7,606	2·3	0·05	0·77	NS		
7. *Str.* type 12 organism	14	13,271	4·1	0·001	1·4	NS		
8. *Str.* type 12 CHO	14	19,483	6·0	0·001	2·0	0·05	1·5	NS
9. *Str.* type 12 M prot.	8	9,699	3·0	0·01	0·98	NS	0·73	NS
10. *Str.* type 14 organism	8	6,940	2·1	0·05	0·71	NS	0·52	0·005
11. *Str.* type 24 organism	8	9,634	3·0	0·01	0·98	NS	0·73	0·05
12. *Str.* indiff. organism	8	4,264	1·3	NS				
13. *Str.* gp. D organism	6	3,619	1·1	NS				
14. *Str.* 14A organism	6	2,566	0·79	NS				
15. *Str.* gp. C organism	8	6,328	1·9	NS				

NS, Not significant.

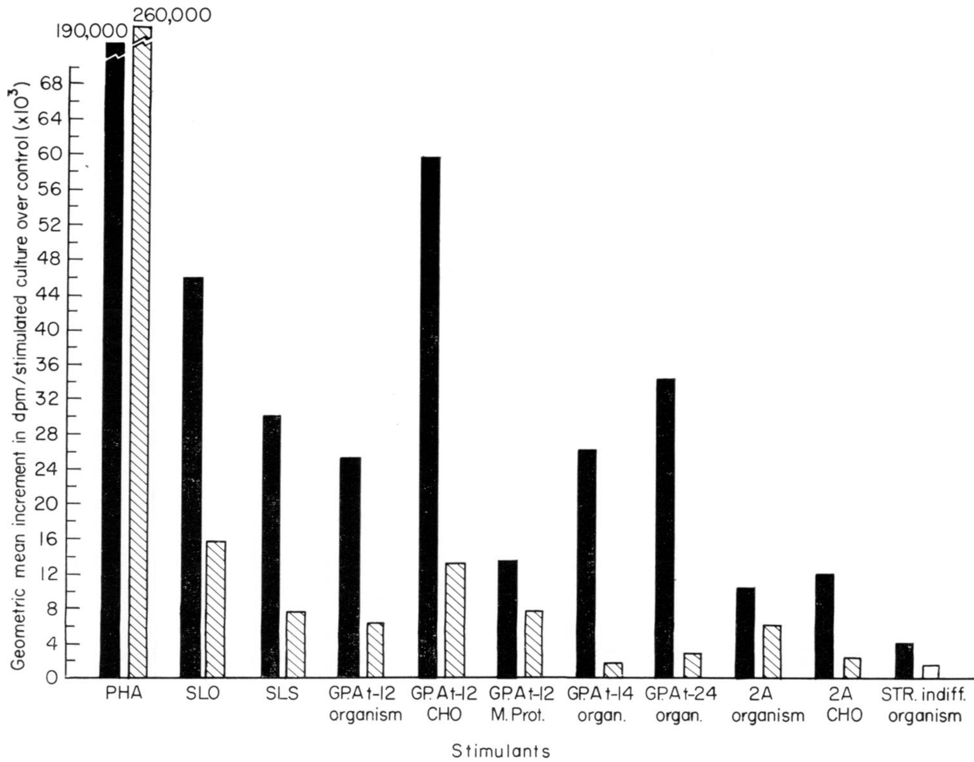

FIG. 2. Illustrates the comparison of the *in vitro* leucocyte culture response to the various stimuli between normals (solid columns) and patients with rheumatic heart disease (hatched columns). Although the response to all the stimulants is decreased in the rheumatic heart patients (except to PHA), those significantly decreased are: *Str.* gp.AT-12 ($P<0.05$), *Str.* gp.At-12 CHO ($P<0.005$), *Str.* gp.AT-14 ($P<0.005$), *Str.* gp.AT-24 ($P<0.01$), *Str.* 2A CHO ($P<0.025$).

TABLE 5. Comparisons of significance between the patient populations

Antigen	Geometric mean dpm		P-value
	Normal	Aphthous	
Str. 2A	18,328	8,678	0·025
	Normal	Rheumatic heart	
Str. gp. A T12	46,544	13,271	0·05
Str. gp. A T12 CHO	75,863	19,483	0·005
Str. gp. A T14	35,150	6,940	0·005
Str. gp. A T24	41,976	9,634	0·01
Str. 2A CHO	19,169	7,606	0·05
Str. gp. C*	7,334	6,328	0·025
Smallpox*†	3,574	3,254	NS

* Not illustrated in Fig. 2.
† Data obtained from 6-day cultures.

The plasma switching experiments were done with the normal-aphthous and normal-rheumatic heart groups to investigate whether any serum factors were responsible for the patients' selective defects in lymphocyte transformation. The technique involved was to wash the cells from two groups, i.e., normals and rheumatic heart patients, and then resuspend the cells in their own or the other subjects' plasma (appropriate controls being used)

TABLE 6. Effect of interchanging normal and RHD patients' plasma on cultured lymphocytes

Antigen	Experiment No.	Normal lymphocytes		RHD lymphocytes	
		Normal plasma	RHD plasma	Normal plasma	RHD plasma
Streptococcal T12 organisms	1	10,220*	11,499	45,646	49,557
	2	67,748	74,003	1,264	742
	3	6,908	2,731	12,494	20,398
	4	44,890	132,206	—	31,430
	5	35,243	38,950	79,569	27,497
	6	123,415	28,495	9,658	1,695

* Mean TdR^3H incorporation of duplicate cultures in dpm.

TABLE 7. Effect of interchanging normal and aphthous stomatitis (AS) patient's plasma on *in vitro* lymphocyte proliferation

Antigen	Experiment No.	Normal lymphocytes		AS lymphocytes	
		Normal plasma	AS plasma	Normal plasma	AS plasma
Str. sanguis 2A organisms	1	19,986*	8,653	6,745	3,693
	2	65,411	127,363	20,674	3,456
	3	2,486	10,997	7,038	14,440
	4	17,005	4,901	10,621	8,122
	5	14,544	9,530	4,790	4,881
	6	2,488	4,461	2,724	6,681
	7	1,312	1,222	5,512	11,884
	8	40,273	15,011	1,937	2,867
	9	5,901	5,145	2,755	12,908

* Mean TdR^3H incorporation of duplicate cultures in dpm.

prior to antigen stimulation. In most instances there was no improvement in the response of the patients' hyporeactive lymphocytes when cultured in normal plasma, nor any suppression of the normal lymphocyte response to antigens by the patients' plasma. There were, however, several exceptions (see Tables 6 and 7).

DISCUSSION

Antigens from the human pathogenic strains of streptococci, i.e. group A types 12, 14, and 24, stimulated a greater degree of lymphocyte transformation in peripheral leucocyte

cultures from most human subjects than did the non-pathogenic streptococci. Halbert's studies strongly imply that the incidence of clinical and subclinical streptococcal infection in the general population is quite high (Halbert, 1964). Lawrence (1952, 1953) has shown that 80–90% of normal individuals have a positive delayed skin test to heat-killed haemolytic streptococci and that this delayed reactivity can be passively transferred with peripheral human leucocytes. Lancefield (1951) has shown that normal individuals can maintain detectable levels of streptococcal type specific antibodies for many years following a specific streptococcal infection. These observations are supported by the work of Fox, Wittner & Dorfman (1966) who were able to elicit transient delayed cutaneous reactivity in 80% of ninety-one adults and 8% of fifty-nine infants skin tested with M proteins from group A streptococci of types 12, 14 and 24. M protein is found in the cell walls of these group A streptococcal strains, but not in the non-pathogenic strains. It serves to delay opsonization and thus enhances their virulence (Lancefield, 1962). This also results in prolonged extra-cellular contact as well as their increased pathogenicity and may be responsible for the greater degree of sensitization of human lymphocytes by group A streptococcal antigens. In our previous studies (Francis *et al.*, 1967) we concluded that the considerably greater *in vitro* human lymphocyte response to pathogenic streptococci and much lower degree of response to non-pathogenic streptococcal antigens probably reflected an 'environmental sensitization' to these organisms. This is supported by the observation that the majority of human cord blood leucocyte cultures are not transformed by SLO or group A type 12 CHO (Leiken & Oppenheim, unpublished observations).

Of particular interest in this paper was the result achieved with the *Str.* strain 2A antigen in the aphthous group. One would expect that since the organism elicited greater delayed hypersensitive reactivity in aphthous patients than in normals, as has been reported by Graykowski *et al.* (1966), it should have stimulated greater reactivity on the part of the aphthous than normal lymphocytes. However, the aphthous lymphocytes were significantly and specifically *hyporesponsive* to *Str.* 2A (Table 2). These apparently contradictory observations can be reconciled only if the increased cutaneous hypersensitivity of the aphthous patients was due to a combination of delayed and arthus reactions.

In view of the selective defect in the lymphocyte response of the apthous patients to *Str.* 2A (Table 5), a group of RHD patients with documented post streptococcal sequellae was studied. If the aphthous patients' depressed response to the *Str.* strain 2A was related to their role in this disease, then a group of patients with rheumatic heart disease could also be predicted to have a comparably reduced lymphocyte response to the Lancefield group A strains.

The differences in the response of the normals and patients with rheumatic heart disease was striking. The lymphocyte response of the RHD subjects to all of the group A strains used in the study plus the *Str.* 2A CHO and *Str.* group C were significantly lower than those of the normal cultures (Fig. 1 and Table 5). This effect did not appear to be type specific or group specific (with reference to the *Str.* group C). However, the response of the RHD patients to PHA and smallpox vaccine was not significantly different from normal.

Antigen stimulated lymphocyte transformation is considered to be a model of cellular immunity (Oppenheim, 1968). Using this assay, Lehner (1964, 1967b) was able to show in patients with recurrent oral ulcerative disease an increase in haemagglutinin titres and increased lymphocyte transformation to saline extracts of oral mucosa. Hirschhorn *et al.* (1964) have shown that lymphocytes from normals were nonspecifically stimulated by

Streptolysin S, but that this response was suppressed in patients with acute rheumatic fever. Oettgen *et al.* (1966) demonstrated a lack of response of tonsillar lymphocytes to Streptolysin O representing, perhaps, an immunologic unresponsiveness to antigens (streptococcal) almost constantly present. Also, Pachman & Fox (1968) showed significant stimulation of DNA synthesis *in vitro* to purified streptococcal M proteins (types 1, 3, 5 and 12) in a group of normals demonstrating varying degrees of immediate or delayed type skin reactivity to these antigens. They also noted that the haemagglutinin titres in the serum of these patients to the specific serotypes were unrelated to lymphocyte stimulation or to delayed cutaneous reactivity. Newberry *et al.* (1968) demonstrated that patients with chronic histoplasmosis showed a depressed synthesis of DNA *in vitro* to histoplasmin which resembled that seen in control patients negative to histoplasmin. However, patients with acute histoplasmosis and positive histoplasmin donor controls showed good *in vitro* DNA synthesis to histoplasmin. It should be pointed out here that this depressed DNA synthesis *in vitro* appears to be purely a cellular phenomenon since plasma switching experiments in our study and that of Newberry *et al.* (1968) generally showed no apparent effect.

Therefore, it is evident that in certain disease states at least, one can demonstrate a depressed cellular proliferative response to the infectious agent or antigen in question. However, this cellular hyporesponsiveness may or may not be associated with a humoral component. It is also worthy to note here that prolonged chronic illness as was seen with the Behçet patient group in this study does not necessarily alter *in vitro* DNA synthesis.

Several speculative explanations for this relative hyporesponsiveness of these aphthous and rheumatic heart patients' lymphocytes to streptococcal antigens might be proposed. First, this may represent a dose response phenomenon since the concentrations of organisms used to produce optimal stimulation of the leucocyte cultures was established only in normal human cultures. Although similar dose–response curves were not established for each patient group, the fact remains that for the doses used, there was a significant difference between the patients and controls. Indeed, preliminary experiments now in progress indicate that patients with rheumatic heart disease have a delayed rate of response as compared to normals.

Secondly, this altered response may be due to immune deviation. It has been shown that when PPD is administered intravenously prior to sensitization with Freund's complete adjuvant antibody production is increased, but that both delayed hypersensitivity and *in vitro* lymphocyte transformation with PPD are reduced (Loewi, Temple & Vischer, 1968). Similarly, it has been shown that antibody titres to group A streptococcal antigens are elevated in the RHD patients (Dudding & Ayoub, 1968). Therefore, the persistence of streptococcal antigens in the tissues of the RHD patients may have promoted antibody production at the expense of delayed hypersensitivity and *in vitro* lymphoproliferative responses of these patients to these antigens. This phenomenon has also been termed 'split tolerance' by some workers, and the actual mechanism for this cellular hyporesponsiveness may be due to high dose tolerance due to chronic exposure to high doses of antigen. In relation to this line of reasoning, it has been shown by Hirschhorn *et al.* (1964) that during acute rheumatic fever, human peripheral lymphocyte response to SLS is selectively depressed; possibly because these individuals lack the ability to neutralize the SLS or the reactive sites on the lymphocyte cell surface are already bound by *in vivo* antigen. Since streptococcal antigens are known to be retained in tissues for long periods of time (Humphrey & Page, 1949), perhaps the slow sustained release of large amounts of streptococcal antigen from

tissue deposits keeps the majority of the reactive cell sites bound with streptococcal antigen. These 'coated' or partially paralyzed lymphocytes would not be as reactive to a streptococcal antigen stimulus in cell culture as would a normal 'uncoated' cell. At the present time, however, we have no proof for these hypotheses.

Third, there may be a familial tendency to rheumatic fever (Wilson & Schweitzer, 1954) and consequently to rheumatic heart disease. There is strong evidence to indicate that multiple recurrences of rheumatic fever culminate in rheumatic heart disease (Taranta, 1964). Studies by Humphrey & Page (1949) support the view that there may be a difference in the manner in which individuals degrade cellular components of group A streptococci. Perhaps these individuals lack genetic information that is necessary at a cellular level to inactivate or degrade these streptococci. This would lead to an increased susceptibility to frequent and prolonged streptococcal infections and sequelae.

Finally, it is possible that the immunocompetent cells committed to respond to the streptococcal antigens in these patients are tissue bound and are involved in reactions at the sites containing antigen. They are, therefore, not as likely to be present in the circulation as normal unstimulated subjects, and their cellular hyporesponsiveness is only a relative rather than an absolute manifestation of their disease. In neither disease do our findings, although suggestive, prove streptococcal aetiology and the decreased cellular response may be an epiphenomenon.

ACKNOWLEDGMENTS

Appreciation is expressed to Miss Constance DeJong, Mrs Shirley Geis and Mrs Frances Davis for their excellent technical assistance, and to Mr Charles Maclean and Miss Rosalyn Singleton for assistance with the statistical analysis, Dr Stephen Epstein of the National Heart Institute for kindly providing patients with rheumatic heart disease, and Dr Isaac Ginsburg for his encouragement and suggestions.

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