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STIMULATION OF HUMAN TONSILLAR LYMPHOCYTES *IN VITRO*

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SUMMARY

We have studied the *in vitro* behaviour of cultured human tonsillar lymphocytes. In comparison with peripheral blood lymphocytes these cells show a higher degree of formation of large cells and mitoses in control cultures without any additive. They behave in a manner similar to peripheral blood lymphocytes when cultured with phytohaemagglutinin (PHA), streptolysin S (SLS) and specific antigens. The only exception is a lack of response to streptolysin O (SLO).

Human peripheral blood lymphocytes have been studied *in vitro* by an increasing number of investigators since it was discovered in 1960 (Nowell, 1960a) that phytohaemagglutinin (PHA), an extract of *Phaseolus vulgaris*, causes these cells to change into larger cells, and to undergo mitosis. This effect is most pronounced after an incubation period of 3 days. The mechanism of action of phytohaemagglutinin is not understood; some form of immunological reaction has been suspected. It has been observed by others (Nowell, 1960b; Schrek & Rabinowitz, 1963; Robbins, 1964) and ourselves (Hirschhorn *et al.*, 1964a) that only few of the peripheral blood lymphocytes from patients with chronic lymphatic leukaemia are so changed. The suggestion has been made that phytohaemagglutinin stimulates the production of γ-globulin and of antibody (Bach & Hirschhorn, 1963; Elves *et al.*, 1963; Forbes, 1965) by lymphocytes. An increase in RNA-synthesis has been convincingly documented (Cooper & Rubin, 1965).

A similar response of cultured peripheral blood lymphocytes has been shown to occur when they are exposed to streptolysin S (SLS) (Hirschhorn *et al.*, 1964b). This response is almost as pronounced as that found with phytohaemagglutinin, and is diminished only in cells from patients with acute rheumatic fever, which, however, retain their ability to respond to phytohaemagglutinin.

A lesser degree of cell enlargement and mitotic activity has been observed when peripheral

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blood lymphocytes from immunized donors were cultured in the presence of specific antigens, such as tuberculin, diphtheria toxoid or pertussis vaccine (Pearmain, Lycette & Fitzgerald, 1963; Hirschhorn *et al.*, 1963). Generally, this effect is at its peak after a culture period of 5 days and is seen in only 5–35% of the lymphocytes, while 80–90% respond to phytohaemagglutinin.

In recent years, on the basis of clinical and experimental evidence, the concept of 'central lymphoid tissues' as contrasted to 'peripheral lymphoid tissues' has been proposed (Good, Peterson & Gabrielsen, 1964). The former, including the thymus and, possibly, other gut-associated lymphoid tissue—Peyer's patches, appendix, tonsils, adenoids—has been thought to function in the ontogenetic development of lymphoid tissues and immunologic capacity but to be less active in day-to-day immunologic functions than the latter, mainly the lymph nodes and the spleen.

It seemed of interest, therefore, to culture lymphoid cells from human tonsils, to study their response *in vitro* to phytohaemagglutinin, streptolysin S and specific antigens, and to compare these effects with those observed with peripheral blood lymphocytes.

MATERIALS AND METHODS

Nineteen pairs of tonsils, obtained from children between 4 and 12 years of age undergoing routine tonsillectomy, were evaluated. The tonsils were placed on ice immediately following removal and processed within 2 hr. They were washed with Eagle's minimal essential medium modified for suspension culture (MEM-S) (Eagle, 1959) containing penicillin and streptomycin, minced with scissors, and passed through the wire mesh filter of a blood transfusion set. The cells were washed in three changes of medium, centrifuged at 100 *g* for 5 min after each washing, and suspended in MEM-S with 20% foetal calf serum and 1% of 200 mM L-glutamine, containing 100 units penicillin and 100 μg streptomycin per ml. The final concentration of cells was 1 000 000 per ml. Cultures were set up in 4 ml replicates in plastic tubes and incubated at 37°C.

Cultures of peripheral blood lymphocytes were prepared as described in detail previously (Hirschhorn, 1965). In some experiments the limited amount of blood available made necessary the use of a micro-method in which 0·2 ml of heparinized venous blood were added to 4 ml complete medium in each culture tube (Hirschhorn, 1965).

Phytohaemagglutinin (Difco, type M) was added at a concentration of 0·1 ml per 4 ml of culture. Streptolysin S and streptolysin O, prepared by the method of Bernheimer (Bernheimer, 1949; Weissmann, Keiser & Bernheimer, 1963) were added at 100 or 20 haemolytic units in 0·1 ml per 4 ml of culture. Polio vaccine type 1, 2 and 3 (Pfizer), alumephosphate absorbed diphtheria toxoid (Parke Davis), tetanus toxoid fluid (Lilly), pertussis vaccine (Parke Davis) and second strength tuberculin purified protein derivative (Merck, Sharp & Dohme) were added undiluted or diluted 1:10 in 0·1 ml per 4 ml of culture.

The cultures were harvested after varying periods as indicated at 4 hr following the addition of 0·05 μg Velban (Lilly) in 0·1 ml, exposed briefly to a solution of 1% sodium citrate in water, fixed in a mixture of 3 parts ethanol and 1 part glacial acetic acid and stained on cover slips with 0·5% acetic orcein. The response was judged by classifying at least 1000 cells as small lymphocytes, large cells, or mitotic figures. The degree of response was defined as the percentage of large cells plus mitoses.

RESULTS

Data concerning the degree of stimulation of tonsillar lymphocytes after a culture period of 3 and/or 5 days are presented in Table 1. Each line represents a different pair of tonsils. There was marked variability in the control cultures without any additive. The percentage of enlarged cells plus mitoses ranged between 2 and 31% after 3 days and between 4 and 39% after 5 days. The addition of PHA produced a relatively uniform increase in response at both Day 3 and Day 5, regardless of whether few or many cells were enlarged in the control cultures. The response to SLS was similar, although slightly less in most instances than that seen with PHA.

TABLE 1. Response of tonsillar lymphocytes to phytohaemagglutinin (PHA) and streptolysin S (SLS). (Each line represents the results obtained with a different pair of tonsils)

Large cells and mitoses (%)					
Day 3			Day 5		
Control	PHA	SLS (100 units)	Control	PHA	SLS (100 units)
13	62				
31	57	71	16	66	74
21	65	51			
18	78	66	4	80	72
26	60	61	39	78	75
2	69	66	7	67	61
			29	72	64
			39	81	58
			6	76	56
			8	77	52
21	58	60			
12	72				
			27	79	47
			16	74	
			28	72	
			30	66	
			25	69	
			6	50	
			20	55	

Tonsil lymphocyte cultures were not sterile. With penicillin and streptomycin in the medium, however, slight bacterial contamination usually did not prohibit short-term lymphocyte culture. Only occasional cultures had to be discarded because of massive contamination.

Some degree of stimulation is seen as early as 24 hr after the culture has been started with both PHA and SLS, as is shown in Fig. 1. In this experiment cells from a single tonsil were followed through a culture period of 8 days. Maximal stimulation by PHA and SLS was seen on the 6th day. However, the differences observed between the 4th and the 8th day were

very small. A lesser but definite initial increase in the number of large cells and mitosis was also seen in the control cultures, but from the 3rd day of culture a plateau was established which remained lower than the corresponding values of PHA or SLS-treated cultures.

Results of an experiment comparing the respective responses of tonsillar and peripheral blood lymphocytes to PHA and SLS are summarized in Table 2. While the maximal response

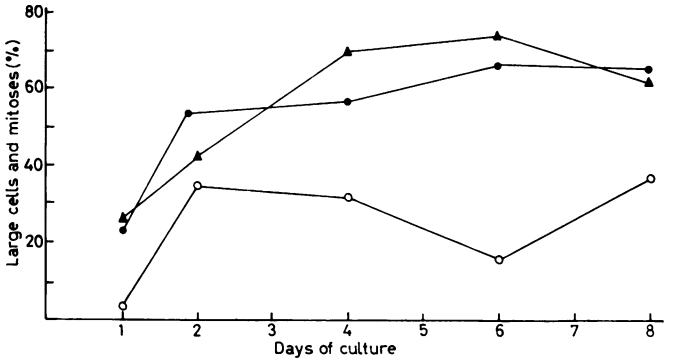

FIG. 1. Time curve of response of tonsillar lymphocytes to phytohaemagglutinin (●) and streptolysin S (▲). ○, Control.

TABLE 2. Comparison of response to tonsillar and peripheral blood lymphocytes to phytohaemagglutinin (PHA) and streptolysin S (SLS)

Patient	Culture	Large cells and mitoses (%)			
		Day 3		Day 5	
		Tonsil	Blood	Tonsil	Blood
1	Control	18	ND	4	1
	PHA	78	ND	80	86
	SLS	66	ND	72	80
2	Control	26	4	39	3
	PHA	60	77	77	81
	SLS	61	31	75	69
3	Control	2	5	7	4
	PHA	69	75	67	87
	SLS	66	25	61	88

ND = Not done.

to PHA was seen after a culture period of 3 days with both tonsillar and peripheral blood lymphocytes, the degree of stimulation by SLS observed with peripheral blood lymphocytes was lower after 3 days of culture than after 5 days of culture. In contrast, with tonsillar lymphocytes, stimulation after three days of culture was not significantly less than after 5 days.

TABLE 3. Absorption of streptolysin S (SLS) with tonsillar lymphocytes

Added to 24 hr regular culture		Large cells and mitoses (%)
Nil		27
PHA		79
SLS 100 units		48
SLS 20 units		10
SLS 100 units, 24 hr at 37°C		42
SLS 20 units, 24 hr at 37°C		18
Supernate from 4 ml 24 hr cultures		
I	A	26
	B	12
	C	11
II	A	35
	B	58
	C	82
III	A	23
	B	35
	C	58

I, No SLS added; II, 100 units SLS added at time 0; III, 20 units SLS added at time 0.

A, Pre-incubation with 14×10^6 cells/ml; B, pre-incubation with $3{\cdot}5 \times 10^6$ cells/ml; C, pre-incubation with $0{\cdot}75 \times 10^6$ cells/ml.

TABLE 4. Response of tonsillar and peripheral blood lymphocytes to specific antigens

	Large cells and mitoses (%)			
	Patient L.S.		Patient A.C.	
	Tonsil	Blood	Tonsil	Blood
Control	22	6	27	11
PHA	73	ND	67	ND
Diphtheria	15	7	59	23
Polio	69	22	55	30
Pertussis	42	19	41	28
Tetanus	39	25	50	32

ND = Not done.

In Table 3 the following experiment is summarized. Cultures were set up with lymphocytes from the same tonsil at various cell concentrations, with and without SLS. Following an incubation period of 24 hr, the supernatant fluid of these cultures was added to regular cultures of cells from the same tonsil, 24 hr old. It is evident that incubation of SLS alone for 24 hr at 37°C did not alter its activity, but that exposure to an increasing number of lymphocytes during this incubation period progressively reduced the effectiveness of SLS upon regular cultures of tonsillar lymphocytes.

Incubation of the tonsillar lymphocytes with specific antigens for 5 days produced significant stimulation in several instances (Table 4). The cells from patient L.S. showed a response to polio vaccine, pertussis vaccine and tetanus toxoid, but no response to diphtheria toxoid. The cells from patient A.C. showed a positive response to all four of these antigens. These children had in the past been immunized with these antigens. Peripheral blood lymphocytes from the same patients, incubated with the same antigens for 5 days, showed similar results, although the degree of stimulation was not necessarily identical.

Lymphocytes from five tonsils were cultured for periods from 1 to 8 days in the presence of 100 units of SLO. No stimulation was observed beyond the large cells seen in control cultures.

DISCUSSION

Stimulation of peripheral blood lymphocytes *in vitro* has been reported with a variety of specific antigenic and non-specific agents. Although presumptive evidence is available that these cells are capable of antibody production *in vitro*, the possibility must be considered that these reactions represent the *in vitro* equivalent of cellular or delayed type hypersensitivity. Similar behaviour of cells derived from 'central' lymphoid tissue would strengthen the probability that *in vitro* stimulation produces true immunologic responses. The tonsil has recently been tentatively included in the 'central' lymphoid tissue (Good *et al.*, 1964), and provides a readily available source for study.

The number of large cells and mitoses arising 'spontaneously' in cultures of tonsillar lymphocytes is significantly higher than the 5–15% of stimulated cells found in control cultures of peripheral blood lymphocytes (Hirschhorn *et al.*, 1963). Since the tonsil represents the lymphoid tissue exposed at all times to external antigen (e.g. bacteria), this apparently spontaneous change may be the result of stimulation initiated *in vivo*. It must also be taken into consideration that these tonsillar cultures are rarely sterile and that therefore they contain bacterial antigens to which the patients have been previously exposed. Evidence for this possibility comes from the difference in the results obtained when the cells are cultured with SLO or with other bacterial antigens. Since SLO is an antigen of one of the most common bacteria to invade the tonsil, the lack of response may represent either the result of maximal stimulation by this antigen in the control cultures or immunologic unresponsiveness to an antigen which is almost constantly present. The fact that tonsillar lymphocytes are capable of responding to specific antigens to which the patient has been sensitized is demonstrated in the results. It is of particular interest that 'central' lymphatic tissue behaves in culture in the same way as do peripheral blood lymphocytes when exposed to specific antigens.

The uniform increase of the number of large cells and mitoses upon the addition of PHA indicates that the small lymphocytes in the tonsil is capable of non-specific derepression in

a similar manner as is the small peripheral blood lymphocyte. The possible mechanism of this derepression has been discussed in detail elsewhere (Hirschhorn & Hirschhorn, 1965). One of the initial events in the stimulation of the blood lymphocytes is a rapid and marked increase in RNA-production (Cooper & Rubin, 1965). We have found a similar increase in RNA-production in the tonsillar lymphocytes cultured with PHA. The detailed results of these experiments will be reported at a later date.

SLS, a non-antigenic product of the group A β-haemolytic streptococcus, capable of producing non-specific stimulation of peripheral blood lymphocytes (Hirschhorn *et al.*, 1964b), has a similar effect on the tonsillar cells. In both cell systems, SLS produces on the average a somewhat smaller increase in the number of large cells and mitoses than does PHA, but the increase is greater than that produced by specific antigens. SLS, a substance capable of producing tissue damage by means of release of lysosomal enzymes (Weissmann *et al.*, 1963), has been implicated as the possible etiologic agent of rheumatic fever (McLeod, 1959). Since the portal of entry of this substance is via the upper respiratory lymphatic tissue including the tonsil, the observed stimulation of tonsillar lymphocytes by SLS may represent binding of this substance, thereby preventing its entry into the blood stream. Our findings of the increasing partial absorption of the stimulatory capacity of SLS by pre-incubation with increasing numbers of tonsillar lymphocytes would tend to support this theory. The observation (Hirschhorn *et al.*, 1964b) that blood lymphocytes from patients with acute rheumatic fever show a very poor response to SLS may therefore represent an inability to bind this substance adequately in the lymphatic tissue, thereby permitting its entry into the blood stream and the subsequent tissue damage associated with rheumatic fever.

In view of the fact that tonsils provide an easily obtainable source of large numbers of lymphocytes from a single individual, and since the tonsillar lymphocytes appear to behave in culture identically to the peripheral blood lymphocyte, it should be possible to derive more accurate information about the metabolism and mechanisms of the *in vitro* response. We have already begun to utilize the tonsil system for the study of RNA-metabolism, and it is hoped that such questions as γ-globulin production may be more easily resolved using this tissue.

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