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MIKULICZ'S RECURRENT ORAL APHTHAE: THE EFFECT OF ANTI-LYMPHOCYTE SERUM UPON THE *IN VITRO* CYTOTOXICITY OF LYMPHOCYTES FROM PATIENTS FOR ORAL EPITHELIAL CELLS

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SUMMARY

Preincubation with rabbit anti-human lymphocyte serum suppressed the *in vitro* cytotoxicity of peripheral blood lymphocytes from patients suffering from Mikulicz's recurrent oral aphthae for oral epithelial cells. Further studies revealed that although the anti-lymphocyte antibody remained bound to the lymphocytes after the pre-incubation procedure it exerted no demonstrable cytotoxic effect and induced a minimal degree of transformation. The mechanisms by which the anti-lymphocyte serum may have suppressed the lymphocytotoxicity are discussed.

INTRODUCTION

Recently it has been shown that peripheral blood lymphocytes, but not sera, from patients suffering from Mikulicz's recurrent oral aphthae were cytotoxic for human oral epithelial cells in tissue culture (Dolby, 1969). It was concluded that Mikulicz's recurrent oral aphthae may be a disease in which a hypersensitivity reaction involving the small lymphocyte adversely affects the oral epithelium.

The *in vitro* cytotoxic effect of lymphocytes upon allogeneic cells has been prevented by preliminary incubation of the lymphocytes with anti-thymus serum produced in the rabbit (Levey & Medawar, 1966) and the horse (Shorter *et al.*, 1968). In this investigation the effect was determined of anti-human lymphocyte serum prepared in the rabbit upon the *in vitro* cytotoxicity of lymphocytes from patients suffering from MROA for oral epithelial cells. At the same time, the cytotoxicity, mitogenic effect and antibody binding capacity of the rabbit anti-human lymphocyte serum in relation to human lymphocytes was examined.

MATERIALS AND METHODS

Source and preparation of suspensions of lymphocytes. Peripheral blood lymphocytes were

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obtained from six patients who suffered from Mikulicz's recurrent oral aphthae (three women and three men, age range 20–61) and from six healthy subjects (three women and three men, age range 23–61) who experienced no recurrent oral ulceration. The lymphocytes were separated from the venous blood by the method previously described (Dolby, 1969), and were finally suspended in TC 199 tissue culture medium in a concentration of 1×10^6 cells/ml. Viability of the lymphocytes, assessed by trypan blue exclusion, exceeded 90% in each instance.

Preparation of suspensions of oral epithelial cells. Fresh specimens of gingiva were obtained from patients undergoing surgical periodontal treatment. The cell suspensions were prepared in the manner described previously (Dolby, 1969) and the cells were suspended in TC 199 in a final concentration of $2–8 \times 10^5$ cells/ml. No cell suspensions were used in which the viability, estimated by trypan blue exclusion, did not exceed 70%.

Preparation of rabbit anti-human lymphocyte serum and normal rabbit serum. Peripheral blood lymphocytes were obtained from volunteers using the method previously described (Dolby, 1969). After being washed twice in TC 199 the lymphocytes were resuspended in 2 ml of a sterile saline solution at a concentration of 3×10^6 lymphocytes/ml. Rabbits were injected by the marginal ear vein with the lymphocyte suspension on two occasions at an interval of 2 weeks and were bled 1 week after the second injection. Normal rabbit serum (NRS) was obtained from rabbits of similar age and breed which had not been immunized. The serum was absorbed twice with twice the volume of AB and O red cells.

Assessment of cytotoxicity of the rabbit anti-human lymphocyte serum for human lymphocytes. The cytotoxic effect of the rabbit anti-human lymphocyte serum (RAHLS) was determined by a modification of the methods of Sanderson (1964) and Wigzell (1965). Cell suspensions were adjusted to a concentration of 2×10^6/ml in TC 199 and 10% foetal bovine serum. [^{51}Cr]Sodium chromate (Radiochemical Centre, Amersham; specific activity 50–150 mCi/mg) was added and the suspensions incubated at 37°C for 30 min while the container was rotated at 15 rev/min. After labelling, the cells were washed until free ^{51}Cr in the supernatant was minimal and finally suspended at a concentration of 1×10^5/ml in TC 199 medium. To each ml, 0·1 ml of inactivated (56°C for 30 min) or fresh RAHLS or NRS was added. The tubes were incubated at 37°C for 1 hr and the total radioactivity of the samples and of equal aliquots of the cell free medium was determined.

Mitogenic activity of the rabbit anti-human lymphocyte serum. 0·1 ml of RAHLS was added to 1 ml cultures containing 10^6 lymphocytes in TC 199 with 20% foetal bovine serum. The lymphocytes were removed from the medium after 1 hr, washed three times in TC 199 and resuspended in TC 199 with 10% FBS and 10% NRS or TC 199 with 10% FBS and 10% RAHLS. The cultures were gassed with 5% CO_2/air mixture and cultured for 24 hr when the second group of cultures was again washed three times in TC 199 and resuspended in TC 199 with 10% FBS and 10% NRS. At 72 hr [2-^{14}C]thymidine (Radiochemical Centre, Amersham; specific activity 16 mCi/mM) 0·3 mCi/ml of culture, was added and 6 hr later the cells washed three times in ice cold saline solution. ^{14}C-DNA labelled protein was precipitated by the addition of 5% trichloracetic acid. The precipitates were redissolved by heating at 90°C for 30 min and then prepared for counting by the addition of Bray's naphthalene scintillator solution. The results are expressed as counts per minute per million lymphocytes.

Antibody binding for lymphocytes. Antibody binding was detected with a modification of the method described by Moller (1964). Lymphocyte suspensions (1×10^6/ml in TC 199

and 10% FBS) were incubated in 10% RAHLS for 1 hr. Samples of the suspensions were removed and washed three times in buffered saline solution (BSS). The supernatant was removed from both the washed and unwashed samples and replaced with 0·1 ml of fluorescent goat anti-rabbit globulin (Difco, Chadwell Heath, Essex). The cells were brought into suspension by shaking and incubated at 37°C for 15 min. After incubation all the samples were washed three times in 1 ml BSS, resuspended to their original volume in BSS and examined with a Zeiss microscope with ultraviolet source (BG 12 filter).

Suppression of in vitro cytotoxicity of lymphocytes. The oral epithelial cells ($2–8 \times 10^5$ per ml) were incubated at 37°C with 1×10^6 lymphocytes in TC 199 and 10% FBS for 24 hr. In each test the epithelial cells were incubated separately with lymphocytes from a normal healthy subject and with lymphocytes from a patient suffering with MROA. At the end of 24 hr the viability of the oral epithelial cells in each tube was assessed on the basis of trypan blue exclusion and a total count was made using a Fuchs–Rosenthal counting chamber. Additional cultures contained similar mixtures of cells except that prior to their addition to the suspensions of oral epithelial cells, the lymphocytes from normal subjects and from patients suffering from MROA were incubated for 1 hr at 37°C in TC 199 plus 10% FBS containing 10% of either NRS or RAHLS. This was followed by two washings in TC 199. All the cultures were carried out in triplicate.

RESULTS

Assessment of cytotoxicity of the rabbit anti-human lymphocyte serum for human lymphocytes. The percentage release of ^{51}Cr from the labelled lymphocytes after incubation with absorbed inactivated anti-lymphocyte serum (4·9%) did not differ significantly ($P > 0·05$) from the release obtained after incubation with NRS which had also been absorbed (5·3%). The release of ^{51}Cr following 1 hr incubation with absorbed, fresh anti-lymphocyte serum was considerably higher (54·6%).

Mitogenic activity of the rabbit anti-human lymphocyte serum. The uptake of [2-^{14}C]-thymidine by the lymphocytes after 1 or 24 hr of culture with RAHLS or NRS is shown in Table 1.

Antibody binding of the rabbit anti-human lymphocyte serum for lymphocytes. Where lymphocytes had been incubated with 10% NRS faint fluorescence of the cell wall was

TABLE 1. The uptake of [^{14}C]thymidine expressed as cpm/10^6 cells following incubation in anti-lymphocyte serum (ALS) for 1 hr, 24 hr and normal rabbit serum (NRS)

Incubation with ALS 1 hr	Incubation with ALS 24 hr	Incubation with NRS
59·9*	2618·2*	38·4*
245·2	2123·7	47·0
198·0	2821·8	119·0

* Mean of three replicates.

observed. Where the lymphocytes had been incubated with 10% RAHLS brilliant cell wall fluorescence was observed in each experiment.

Suppression of in vitro cytotoxicity by RAHLS. The mean number of epithelial cells surviving following incubation with lymphocytes from normal and aphthous patients with and without incubation with anti-lymphocyte serum is summarized in Table 2 and shown graphically in Fig. 1. Preincubation of aphthous lymphocytes prior to their addition to the oral epithelial cell cultures resulted in a level of survival in these cultures which did not differ

TABLE 2. The mean number of epithelial tissue culture cells remaining after incubation with pretreated (RAHLS) and untreated lymphocytes

	Mean* (±SD) number of epithelial cells × 10^5 remaining after incubation			
Experiment No.	Control	Control+RAHLS	MROA†	MROA+RAHLS
1	0·62±0·01	0·66±0·07	0·46±0·01	0·57±0·09
2	0·34±0·01	0·38±0·01	0·23±0·05	0·32±0·01
3	0·52±0·08	0·52±0·06	0·41±0·06	0·54±0·070
4	0·61±0·06	0·67±0·07	0·42±0·07	0·62±0·03
5	0·55±0·08	0·45±0·01	0·51±0·09	0·56±0·09
6	0·47±0·03	0·60±0·07	0·42±0·02	0·56±0·05

* Average of three tubes.

† Lymphocytes obtained from patients suffering from MROA.

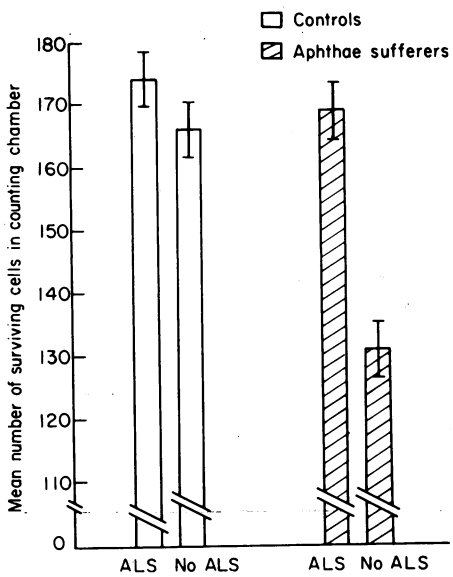

FIG. 1. The mean level of survival (± 2 SE) in the six experiments in which treated and untreated, normal or aphthous lymphocytes were incubated with oral epithelial cells for 24 hr. ALS = Anti-lymphocyte serum.

significantly ($P>0{\cdot}05$) from the oral epithelial cell cultures where normal lymphocytes have been added. The preincubation of normal lymphocytes with anti-lymphocyte serum did not result in any significant ($P>0{\cdot}05$) alteration in the level of survival of the oral epithelial cells.

DISCUSSION

The method by which anti-lymphocyte serum exerts an immunosuppressive effect is unknown, although several theories exist in relation to its mode of action (James, 1967). In the experiments described the RAHLS remained bound after preincubation and may therefore have acted as a 'blindfold' (Levey & Medawar, 1966), preventing recognition by the lymphocytes of other antigens in the mixed culture. Preincubation of the lymphocytes in RHALS for 1 hr resulted in a minimal degree of transformation, considerably less than that achieved following 24 hr incubation in RHALS. Caron (1967) concluded that the period of contact between the lymphocytes and certain specific antigens required to induce transformation is less than 1 min and probably no more than 10 sec. However, there would appear to be a minimal concentration of anti-lymphocyte serum which is required to produce significant levels of transformation (Eijsvoogel *et al.*, 1969). Failure to correlate mitogenic activity with immunosuppressive potency of anti-lymphocyte serum has been previously reported (Greaves *et al.*, 1969).

Preincubation with RAHLS produced considerable agglutination of the lymphocytes which may have profoundly affected the cytotoxic action since close contact is apparently necessary between the lymphocyte and target cell in these reactions (Rosenau, 1963; Wilson, 1965).

Several of the *in vitro* lymphocytotoxic reactions require complement for their completion (Perlmann & Broberger, 1963; Shorter *et al.*, 1968), while others do not (Rosenau & Moon, 1961; Moller, 1965; Watson, Quigley & Bolt, 1966). The *in vitro* cytotoxicity of lymphocytes from patients with MROA for oral epithelial cells occurs in the absence of complement (Dolby, 1969), so that it was possible to employ in these experiments anti-lymphocyte serum with no demonstrable cytotoxic effect. In the experimental situation described by Shorter *et al.* (1968) complement was employed and approximately 25% of the lymphocytes were killed during the course of the experiment. That only a slight but specific cytotoxic effect of anti-lymphocyte serum would suppress such reactions is suggested by the work of Wilson (1965) who concluded that in *in vitro* lymphocyte mediated target cell damage as little as 2% of the lymphocyte population is immunologically active.

In initial experiments in which RAHLS was added directly to the lymphocyte epithelial cell cultures in the ratio of 1 : 10, consistent inhibition of the lymphocytotoxicity was not achieved. Lundren (1969) has recently shown that anti-lymphocyte serum at certain dilutions, will induce a cytotoxic potential in normal non-immunized human lymphocytes against allogeneic fibroblast target cells.

This investigation was undertaken in an effort to confirm the association of the lymphocytes with the cytotoxic effect upon oral epithelial cells in this disease. The suppression achieved supports the concept that it is this cell which is responsible for the target cell damage. Because of the high toxicity of anti-lymphocyte serum (Denman, 1969) its use in relation to this disease would appear to be restricted to such experimental *in vitro* situations.

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