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An increase in peripheral blood Ia-positive T cells in Sjögren's syndrome correlates with a decrease in the autologous mixed lymphocyte response

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SUMMARY

The defective autologous MLR was studied in Sjögren's syndrome (SS) in relation to Ia^+ cells as determined by reactivity with a monoclonal anti-human Ia antibody. By indirect immunofluorescence, the percentage of Ia^+ T lymphocytes was increased in nine of 15 patients. There was no correlation with clinical features or drugs. The percentage of Ia^+ T cells in the non-T cell preparations was normal. An inverse correlation was found between the percentage of Ia^+ T cells and the proliferative response to autologous non-T cells. Removal of Ia^+ T cells enhanced both the autologous MLR and the allogeneic MLR. Thus Ia^+ T cells contain suppressor cells in the MLR, but this may not be the sole explanation for the defective autologous MLR.

INTRODUCTION

The surface markers of the HLA-D region were first characterized on B cells (Winchester *et al.*, 1975). These 'B cell alloantigens' are often referred to as Ia or Ia-like molecules on the basis of many homologies to the murine Ia system. These antigens, also readily demonstrable on monocytes and null cells, are important in regulatory interactions between T and non-T cells (Katz *et al.*, 1975; Niederhuber & Frelinger, 1976; Engleman *et al.*, 1980c). The autologous mixed lymphocyte reaction (AMLR) and allogenic mixed lymphocyte reaction (MLR) provide *in vitro* models for Ia antigen recognition by T cells (Engleman, Benike & Charron, 1980a; Gotlieb *et al.*, 1979).

The presence of Ia molecules on some human T lymphocytes has been demonstrated in several laboratories (Fu *et al.*, 1979; Gotlieb *et al.*, 1979; Reinherz *et al.*, 1979a; Kaszubowski, Goodwin & Williams, 1980; Shurman *et al.*, 1980). There is a small and highly variable number of Ia bearing, circulating T cells (Ia^+ T cells) in normal subjects although some workers found none at all (Reinherz *et al.*, 1979a; Charron *et al.*, 1980). The percentage of circulating Ia^+ T cells is markedly increased in several diseases including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (Yu *et al.*, 1980a). The mechanism responsible for this increase, and the possible role of Ia^+ T cells in pathogenesis, is unknown.

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The expression of Ia antigen could be a common feature of 'activated' T cells as suggested by the finding that mitogenic and allogeneic stimulation of T lymphocytes leads to the synthesis of Ia molecules (Evans *et al.*, 1978; Ko *et al.*, 1979; Yu *et al.*, 1980b). The number of Ia^+ T cells may increase as a consequence of inflammation accounting for their high percentage in sites such as rheumatoid synovial fluid (Yu *et al.*, 1980c). Thus, a correlation between Ia^+ T cells and clinical findings or other immunological abnormalities could yield new information as to the biological significance of Ia^+ T cells.

We have recently found a decreased AMLR in the majority of patients with Sjögren's syndrome (SS) (Miyasaka *et al.*, 1980). The decreased AMLR is unrelated to anti-lymphocyte antibodies or to treatment with corticosteroids. The AMLR is also defective in SLE (Kuntz, Innes & Weksler, 1979; Sakane, Steinberg & Green, 1978a; Sakane *et al.*, 1978b), chronic lymphocytic leukaemia (Smith, Knowlton & Koons, 1977) and Hodgkin's disease (Engleman *et al.*, 1980a). An analogous system, the syngeneic mixed lymphocyte response, is decreased in several autoimmune-susceptible strains of mice (Gilmcher *et al.*, 1980; Hom & Talal, 1982). A decreased AMLR has generally been attributed to the responding cells although there are exceptions (Kuntz *et al.*, 1979; Moody *et al.*, 1979). There has been no previous study of AMLR in relation to Ia^+ T cells.

We studied 15 patients with SS using a well defined monoclonal antibody against human Ia antigens. The number of Ia^+ T lymphocytes was increased in nine patients. Most subjects with this abnormality also showed a poor proliferative response in the AMLR even though the number of Ia bearing cells in the AMLR-stimulating population was normal. There was an inverse correlation between the percentage of Ia^+ T cells and the proliferative response to autologous non-T cells in the AMLR. This led us to investigate possible suppressor cell activity in the Ia^+ T cell population.

MATERIALS AND METHODS

Patient population. Fifteen patients (four males and 11 females, ranging from 27 to 72 years of age) were studied. The diagnosis of SS was based on previously established criteria (Bloch *et al.*, 1965). The presence of keratoconjunctivitis sicca was substantiated by Rose-Bengal staining and abnormal Schirmer test. Salivary insufficiency was established by measurement of parotid flow rate and the presence of lymphocytic infiltration on labial salivary gland biopsy (Greenspan *et al.*, 1974; Daniels *et al.*, 1975). SS was associated with RA (secondary SS) in two patients, with CREST syndrome in one patient and with dermatomyositis in one patient. One patient with RA also had chronic lymphocytic leukaemia. Treatment was symptomatic except for five patients on low-dose steroids (less than 10 mg/day of prednisone). The control group consisted of 12 healthy volunteers (eight males and four females, ranging from 26 to 55 years of age).

Preparation of cell populations. The technique has been described in detail elsewhere (Miyasaka *et al.*, 1980). Briefly, 60 ml of heparinized blood was diluted twice in RPMI 1640 (GIBCO, Grand Island, New York, USA) and layered onto Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, New Jersey, USA). After centrifugation at 400 *g* for 30 min, the mononuclear cells were collected from the interface, washed three times, and resuspended in RPMI 1640 supplemented with 20% inactivated fetal calf serum (FCS) (GIBCO).

The mononuclear cell suspension was plated on FCS coated Petri dishes (No. 1007, Falcon Plastics, Oxnard, California, USA). They were incubated for 1 hr at 37°C in 5% CO_2. Non-adherent cells were harvested by gentle pipetting and processed as indicated below. The adherent cells were washed twice in warm RPMI 1640 and the wash medium was discarded. The cells were released from the Petri dishes by a 15 min incubation at 4°C with 3 mM ethylenediamine tetra-acetic acid (EDTA, Sigma Chemical Co., St Louis, Missouri, USA). They were harvested and washed three times in cold RPMI 1640. The pellet was resuspended at a concentration of 5×10^5/ml in RPMI 1640 supplemented with 10% pooled human serum (PHS). The cells were seeded immediately on microtitre plates (Linbro, Flow Laboratories, Inglewood, California) for study of autologous and allogeneic mixed lymphocyte reactions.

T and non-T cells were separated as described by Sakane *et al.* (1978a). The non-adherent cells were resuspended in RPMI 1640 supplemented with 20% FCS and mixed with neuraminidase-treated sheep erythrocytes (Colorado Serum Co., Denver, Colorado, USA). After a 15 min incubation at 37°C, they were centrifuged at 200 *g* for 10 min and kept at 4°C for 1 hr. The pellet was resuspended gently and layered onto Ficoll-Hypaque. After centrifugation, cells which did not form rosettes with sheep erythrocytes (non-T lymphocytes) were collected from the interface, washed and incubated in FCS-supplemented RPMI 1640 containing 25 μg/ml of mitomycin-C (Mitomycin, Sigma). The suspension was plated on Petri dishes and incubated at 37°C for 30 min. These non-T lymphocytes were washed three times and adjusted to 1×10^6/ml in RPMI 1640 supplemented with 10% PHS.

The cells which formed rosettes (T lymphocytes) were recovered from the Ficoll-Hypaque pellet by lysis of sheep erythrocytes in 0·83% ammonium chloride buffered with trisaminomethane (Trizoma, Sigma). These cells were washed three times and resuspended at 1×10^6/ml in RPMI 1640 supplemented with 10% PHS.

All cell suspensions had a viability greater than 95% by trypan blue dye exclusion. The monocyte suspension contained more than 95% monocytes as evaluated by non-specific esterase positivity and morphology on methyl blue stained smears. The non-T lymphocytes were Ig bearing (85%), esterase negative (80%) and E-rosette-negative cells (95%). The T cell suspension contained more than 95% E-rosette forming cells, less than 3% Ig bearing cells and less than 0·5% esterase positive cells. Contamination by polymorphonuclear cells was always less than 0·5%.

Preparation of antibody 2.06. The characteristics of monoclonal antibody 2.06 have been reported elsewhere (Charron & McDevitt, 1979). This hybridoma product is IgG2b and recognizes a non-allospecific determinant common to all HLA-D types studied. To get large amounts of antibody 2.06, hydridoma cells producing 2.06 in RPMI 1640 were injected intra-peritoneally into BALB/c mice (2×10^6 per animal). Ascites were collected from these mice and screened for positivity.

Identification of Ia positive cells by indirect immunofluorescence with antibody 2.06. A pellet of $1–2 \times 10^6$ T cells, and 0·5–1·0 non-T lymphocytes or monocytes was resuspended in 1:200 dilution of monoclonal antibody 2.06 in 1% bovine serum albumin (BSA). The cells were incubated overnight at 4°C and washed three times in cold medium. A fluorescein conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Cappel Laboratories, Cockranville, Pennsylvania) at 1:10 dilution in 1% BSA was applied to the treated cells for 30 min at 4°C. After three washings, the cells were resuspended in 20–50 μl of 50% glycerol and examined under u.v. epi-illumination (Leitz Orthoplan with Ploem epi-illuminator). At least 200 cells were counted.

Formation of Ia-rosettes. One millilitre of an ox red blood cell pellet was incubated for 1 hr at 37°C in a mixture of 10 ml of chromium chloride ($2{\cdot}5 \times 10^{-4}$ M in saline) and 1 ml of saline containing 0·5 mg of protein A (Pharmacia Fine Chemicals, Piscataway, New Jersey). After three washings, 0·5 ml of the pellet was incubated for 1 hr at room temperature in 0·5 ml of the monoclonal anti-Ia antibody 2.06 (1:40 dilution in 1% BSA supplemented RPMI 1640). After washing, the red cells were adjusted to 1% in the same medium and mixed with an equal volume of T cell suspension (2×10^6/ml). Ia-rosettes were formed and separated as described by Sakane *et al.* (1978a) for E-rosettes. Cell suspensions that were either enriched or depleted of Ia^+ T cells by this rosette method were incubated overnight in RPMI 1640 supplemented with 5% PHS for study of Ia positivity by indirect immunofluorescence.

Removal of Ia^+ T cells. A 1:120 dilution of anti-Ia antibody 2.06 in 0·05 M Tris (pH 9·5) was incubated in Petri dishes for 40 min at room temperature. Dishes were washed four times with RPMI 1640 followed by washing with 1% FCS. Twenty to thirty million purified T cells in 5% FCS were added to the antibody-coated Petri dishes and incubated for 70 min at 4°C. After the first 40 min, the dishes were swirled gently to redistribute non-adherent cells. Following incubation, the dishes were swirled gently and non-adherent cells were aspirated. Non-adherent cells were transferred to fresh antibody-coated dishes and the same procedures repeated once again to remove Ia^+ T cells. No Ia^+ T cells remained (as determined by indirect immunofluorescence) after this procedure.

Mitogen stimulation. Unfractionated cells (2×10^5 in 200 μl) were incubated with optimal doses

of mitogen: 1 μg of purified phytohaemagglutinin (PHA) (Burroughs-Wellcome, Reagent Division, Greenville, North Carolina, USA), 20 μg concanavalin A (Con A) (Miles Yeta, Elkhart, Indiana, USA), or 20 μl (1:10 dilution) of pokeweed mitogen (PWM) (GIBCO). The cells were incubated in complete RPMI 1640 supplemented with 20% FCS for 72 hr at 37°C in 5% CO_2. All cultures were performed in triplicate. One microcurrie of tritiated thymidine (methyl ^{3}H-TdR, specific activity 6·0 Ci/mmoles, Schwarz-Mann, Division of Becton Dickinson and Co., Orangeburg, New York) was added to each well 20 hr before harvesting. The results are expressed as net incorporation (Δc.p.m. for stimulated culture minus background c.p.m. for cultures without mitogen).

Mixed lymphocyte cultures. Triplicate cultures in 200 μl of complete RPMI 1640 supplemented with 10% PHS contained 1×10^5 responding T cells and 1×10^5 stimulating MMC-treated non-T cells or 5×10^4 Mϕ. All cultures were incubated for 7 days at 37°C in 5% CO_2.

Data analysis. Data were analysed on a computer (Wang Laboratories, Inc., Teaksbury, Maine) programmed to reject individual data points more than three standard deviations from the calculated means. Wilcoxen rank sum test and Spearman's rank correlation analysis were used for statistical evaluation.

RESULTS

Increase in Ia⁺ cells in normal subjects after proliferation

Monoclonal antibody 2.06 was selected after many hybridomas were screened for selective binding of their product to various B cell preparations (Charron *et al.*, 1979). The ability of monoclonal antibody 2.06 to also recognize T cell Ia antigens was established in initial experiments. Normal lymphocytes were stimulated by mitogens and by allogeneic or autologous non-T cells. T cells were isolated, by E rosette formation, from control cultures or from stimulated cultures harvested at the time of maximum proliferative response. A comparatively large increase in Ia⁺ T cells was observed as a consequence of the various stimulatory signals (Table 1). Most of these cells were blast-like lymphocytes.

Percentage of Ia⁺ cells in normal subjects

The percentage of Ia⁺ cells was low (mean: 0·85±0·43%) in T cell preparations from healthy volunteers (Table 2). The actual percentage of true Ia⁺ T cells could be somewhat lower, since T cell preparations contained approximately 1% of contaminating Ig⁺ cells. Ia-rosette formation was used to obtain enriched Ia⁺ populations from the T cell fraction in three normal subjects. These enriched populations contained 90% E rosette forming cells and 20–30% Ia⁺ cells by indirect immunofluorescence.

A majority of cells in the non-T lymphocyte and monocyte populations stained brightly by immunofluorescence with monoclonal antibody 2.06 (Table 2). Non-T lymphocytes gave almost the same result (56·3±17·7%) as monocytes (52·6±17·5%), but the latter were often brighter staining. Nevertheless, both populations appeared to be heterogenous in respect to Ia expression because they also contained faintly staining and completely negative cells.

Table 1. Ia positive cells after stimulation of T cell preparations

Nature of the stimulating signal	Mean % Ia⁺ T cells in stimulated culture	Mean % Ia⁺ T cells in control culture
PHA	14·0	
Con A	21·0	0·36
PWM	4·2	
Autologous non-T cells	20·5	
Autologous Mϕ	16·6	0·45
Allogeneic non-T cells	35·0	
Allogeneic Mϕ	15·8	0·45

Table 2. Percentage of Ia^+ cells by indirect immunofluorescence

Patient	T cells	Non-T cells	Mϕ
CA	10·3	59·3	62·0
AL	10·0	nd	nd
WE	9·5	97·6	96·2
HE	8·2	40·0	68·0
RO	6·4	55·0	66·5
SH	4·0	nd	63·2
BA	3·7	45·3	66·5
LU	3·5	nd	79·0
BR	2·1	nd	52·0
CO	1·7	58·8	32·7
VL	1·6	58·2	68·0
SL	1·3	nd	54·5
NE	1·1	61·9	65·0
HA	0·8	nd	52·0
MC	0·7	nd	nd
Normal values (Mean ± s.d.)	0·85 ± 0·42	56·3 ± 13·7	50·6 ± 17·5

nd = not done.

Percentage of Ia^+ cells in Sjögren's syndrome

Nine out of 15 patients had an increased number (> 2%) of Ia T cells ($P < 0·01$) (Table 2). Since the percentage of Ig^+ cells in the T cell preparations averaged only 1·65% (not significantly different from control subjects), the increased number of Ia^+ T cells could not be explained by contamination. The Ia^+ T cells stained faintly like normal T cells. Enrichment by Ia-rosette formation was performed in one patient (RO). The enriched fraction contained 33% Ia^+ cells and 90% E rosettes.

No correlation was found between the percentage of Ia^+ T cells and any clinical parameter or treatment protocol. Patients with increased Ia^+ T cells included six with primary SS and three with associated connective tissue disease. Four were on low-dose corticosteroids.

Ia expression on non-T lymphocytes and monocytes in SS was essentially the same as in normal subjects, both in apparent brightness and in percentage of positive cells.

Correlation of Ia^+ T cells with response to mitogens and to autologous or allogeneic antigens

The proliferative response to PHA and Con A was decreased in some patients but the SS group did not differ significantly from controls. The PWM response was normal (Table 3). There was no correlation between the percentage Ia^+ T cells and the response to phytomitogens.

After stimulation with either non-T lymphocytes or monocytes, the AMLR was decreased in nine patients with SS (Table 4). There was no correlation between the proliferative response and the percentage of Ia^+ cells in either stimulating population. There was an inverse correlation between the AMLR and the percentage of Ia^+ lymphocytes in the responding T cell population (Spearman rank correlation: $P < 0·01$).

The allogeneic MLR was normal in most patients (Table 5). T cell preparations which gave a reduced AMLR proliferated well in response to normal allogeneic non-T cells. The inverse correlation between the AMLR and the percentage of Ia^+ T cells in the responding population suggested the possibility that the Ia^+ T cells were functioning to suppress the AMLR. Accordingly, Ia^+ T cells were removed by a panning procedure from six SS patients and seven control subjects. In general, the AMLR was increased by as much as 50% by this procedure, but this was true both for patients and controls. Moreover, the allogeneic MLR in patients and controls was also increased (up to 71%). Therefore, the Ia^+ T cells may exert some suppressor influence both in AMLR and in

Table 3. Relationship of Ia⁺ T cells to mitogen responses

Patient	% Ia⁺ T cells	Incorporation of ^{3}H-thymidine (Δc.p.m.) after mitogen stimulation		
		Con A	PHA	PWM
CA	10·3	26,678	40,504	25,174
AL	10·0	19,145	38,569	nd
WE	9·5	47,319	64,818	25,846
HE	8·2	17,319	49,337	17,322
RO	6·4	32,925	61,418	15,053
SH	4·0	17,874	26,785	40,760
BA	3·7	8,588	33,937	14,297
LV	3·5	63,826	36,681	35,091
BR	2·1	79,500	66,874	14,203
CO	1·7	58,475	39,603	51,043
VL	1·6	31,841	63,858	21,941
SL	1·3	17,169	27,010	17,169
NE	1·1	46,968	24,203	32,297
HA	0·8	65,767	24,774	27,412
MC	0·7	73,802	122,622	nd
ST	0·6	31,122	41,011	31,122
Normal values (mean s.d.)	0·85 ±0·43	51,794 ±32,298	81,572 ±37,675	28,840 ±15,677

nd = not done.

Table 4. Relationship of Ia⁺ T cells to autologous mixed lymphocyte reaction (AMLR)

Patient	% Ia⁺ T cells	Incorporation of ^{3}H-thymidine (Δc.p.m.) in AMLR	
		T × non-T	T × Mϕ
CA	10·3	1,127	551
AL	10·0	1,304	1,904
WE	9·5	489	3,182
HE	8·2	2,913	1,705
RO	6·4	1,651	584
SH	4·0	4,557	4,960
BA	3·7	1,061	251
LU	3·5	26,257	29,702
BR	2·1	21,001	5,822
CO	1·7	21,259	13,996
UL	1·6	12,372	7,713
SL	1·3	3,432	2,569
NE	1·1	6,009	5,285
HA	0·8	12,501	7,244
MC	0·7	25,783	33,253
ST	0·6	nd	nd
Normal values (mean ± s.d.)	0·85 ± 0·43	19,700 ± 11,854	24,138 ± 14,320

nd = not done.

Table 5. Relationship of Ia^+ T cells to allogeneic mixed lymphocyte reaction

Patient	Ia^+ T Cells	Incorporation of ^3H-thymidine (Δc.p.m.) in allogeneic mixed lymphocyte reaction			
		SS T × normal non-T	SS T × normal Mϕ	Normal T × SS non-T	Normal T × SS Mϕ
CA	10·3	48,073	70,242	32,246	41,186
AL	10·0	61,066	42,198	122,783	79,442
WE	9·5	90,649	90,867	20,778	25,631
HE	8·2	29,582	36,653	14,254	31,406
RO	6·4	17,331	9,829	21,091	11,456
SH	4·0	102,618	29,961	2,492	2,462
BA	3·7	26,065	11,802	6,795	4,813
LU	3·5	72,578	16,530	34,763	15,099
BR	2·1	47,797	51,733	31,111	22,710
CO	1·7	33,579	42,086	33,370	37,638
UL	1·6	61,905	9,653	10,596	4,906
SL	1·3	20,805	20,367	15,219	16,080
NE	1·1	69,461	23,869	33,960	21,172
HA	0·8	29,558	26,375	46,969	9,286
MC	0·7	33,612	67,513	21,073	26,263
ST	0·6	nd	nd	nd	nd

nd = not done.

allogeneic MLR, but this is not limited to the SS patients and cannot be the sole explanation for the decreased AMLR.

DISCUSSION

The number of Ia^+ T cells was increased (>2%) in nine of the 15 SS patients studied. This frequency, as well as the percentage of Ia^+ T cells in individual cases (0·6–10·3%), is similar to the findings in SLE but lower than reported for RA (Yu *et al.*, 1980a). Six of the patients had primary SS which shares an immunogenetic background with SLE (Moutsopoulos, Webber & Valgopoulos, 1979a; Moutsopoulos, Mann & Johnson, 1979b).

There was no correlation between the percentage of Ia^+ T cells and any clinical parameter or treatment regimen. None of our patients received daily doses of steroids >10 mg of prednisone.

Seven of the nine SS patients with decreased AMLR had an increased percentage of Ia^+ T cells. This inverse correlation with Ia^+ T cells raised the possibility that the Ia^+ T cells were suppressing the AMLR. In a different human model (chronic graft-versus-host disease), Reinherz *et al.* (1979b) found that Ia^+ T cells could suppress the proliferative response to *Herpes zoster* in cultures.

The hypothesis that Ia^+ T lymphocytes may contain suppressor cells is supported by the enhancement of AMLR after the removal of Ia^+ lymphocytes from the responder cell suspensions. However, this mechanism seems neither specific nor unique.

The suppression was not restricted to AMLR, as a definite improvement of allogeneic MLR was achieved through the same procedure. Furthermore, a comparable effect was observed in both patients and normal controls.

An increased expression of Ia antigens on conventional T cells subpopulations has been reported in health and disease (Winchester & Kunkel, 1979) but its significance may depend upon the subpopulation to which they belong (Reinherz & Schlossman, 1980). For example, it is widely accepted that murine Ia^+ T cells bearing antigens from the I-J subregion are suppressor cells, though the Ia^+ T cell subpopulation also contains cells responsible for the generation of allogeneic

helper activity (Yu *et al.*, 1980b). In humans Ia antigens are expressed on OKT4⁺ inducer cells upon stimulation by soluble antigen (Reinherz & Schlossman, 1980).

Our study does not preclude the possibility that defects in stimulating non-T cells may also play a part in the decreased AMLR (Smith *et al.*, 1977; Moody *et al.*, 1979). The number and fluorescence intensity of Ia⁺ non-T lymphocytes and monocytes was normal in patients with decreased AMLR. Thus, the decreased proliferative response is probably not due to a lack of triggering Ia antigens on the surface of non-T cells. However, our method does not permit quantification of Ia molecules on the cell membrane, nor does it investigate other surface determinants which may be important for optimal stimulation of autologous T cells (Raff, Picker & Stobo, 1980; Hausman *et al.*, 1980; Hausman, Stities & Stobo, 1981). The decreased proliferation of normal T cells stimulated with non-T cells from four patients with SS is consistent with an earlier result (Miyasaka *et al.*, 1980) and could be relevant to these considerations.

Since T cells and non-T cells are both present in inflammatory infiltrates, an *in vivo* AMLR could provide an explanation for the presence of Ia⁺ T cells in sites of active inflammation such as the rheumatoid synovium (Yu *et al.*, 1980c). Our study confirms that T cells stimulated by non-T lymphocytes express Ia antigens, as reported by others (Evans *et al.*, 1978; Ko *et al.*, 1979; Charron *et al.*, 1980). They also establish that monocytes are effective in inducing Ia synthesis. However, since Ia expression follows T cell proliferation which is impaired in the AMLR in SS, the Ia⁺ T cells in SS may not be generated by an *in vivo* AMLR. Other mechanisms of T cell activation, or possible trapping of AMLR responding cells *in situ*, should be considered. The use of anti-Ia reagents to identify Ia⁺ cells in tissue specimens may permit investigation of this question.

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