IMMUNOLOGICAL T-CELL MEMORY IN THE IN VITRO-INDUCED EXPERIMENTAL AUTOIMMUNE ORCHITIS

Specificity of the Reaction and Tissue Distribution of the Autoantigens*

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Traditionally, it has been postulated that immunological self-tolerance is a result of the elimination of self-reactive lymphocyte clones from the immune system. More recent investigations indicate that this notion does not account for all types of autoantigens. Not only has the mere presence of potentially self-reactive lymphocytes in the normal immune system been demonstrated, but there are now indications that some of these self-reactive lymphocytes may even exert important physiological functions within the normal immune system.

Various lines of evidence led to the conclusion that in many, if not in all cases, foreign antigens can be recognized by T lymphocytes only when their recognition coincides with the recognition of determinants specified by the autologous major histocompatibility gene complex (MHC)[1] (1–3). Jerne (4) and Lindenmann (5) have postulated that the immune system is organized as a network, in which each lymphocyte clone is capable of recognizing antigenic determinants on the antigen-binding structures of other lymphocyte clones. And finally, interaction of thymus-dependent lymphocytes with MHC autoantigens was assumed to be the basis for generation of diversity of the lymphocyte clones (6).

Previous studies suggested that normal rat T lymphocytes can recognize and specifically react against syngeneic embryonic fibroblasts (7), as well as against adult autologous tissues (8, 9). Since the self-reactive lymphocytes could be specifically adsorbed to the syngeneic target cells shortly after being isolated from the donor animal, we concluded (*a*) that the self-reactive T cells are clonally restricted, and (*b*) that these self-reactive clones are normal components of the immune system (10). These studies were carried out with unseparated lymphocyte populations, which made it difficult to determine the nature of the reacting lymphocytes, their fine specificity, and the nature of the self antigens recognized. These limitations would be overcome by selectively enriching the self-recognizing lymphocyte clones for further analysis.

In this article I describe a tissue culture method which permits the functional isolation of self-reactive T-lymphocyte clones. After a procedure which was

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; DMSO, dimethyl-sulphoxide; EAC′, erythrocyte antibody complement; EAO, mixed lymphocyte/autologous testis cultures; EM-H, *N*-2-hydroxyethylpiperazine-*N*′-2-sulfonic acid-buffered Eagle's medium; FCA, Freund's complete adjuvant; FCS, fetal calf serum; 2-ME, 2-mercaptoethanol; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; SRBC, sheep erythrocytes.

first applied for the in vitro generation of specific alloreactive memory T lymphocytes (11–13), we isolated the lymphoblasts that are generated in mixed lymphocyte/autologous testis cultures (EAO), and allowed them to revert back to small lymphocytes (14). These secondary lymphocytes (secondary EAO cells) are functionally selected for their specific reactivity to self antigens. The specificity of the secondary EAO reaction is dictated by the MHC. Only a few distinct cell types can elicit a secondary EAO response, suggesting that the relevant MHC antigens are either restricted to the stimulator cell populations, or that they are recognized in conjunction with tissue-specific non-MHC antigens.

Materials and Methods

Rats. Young adult male rats (8–12 wk) were used throughout all studies. The inbred strains Lewis, AS2, L.AS2, L.BN, and BN were provided by the animal facilities in the Max-Planck-Institute. Strains L.AS2 and L.BN are congenic to Lewis, deriving their genetic background from Lewis, but their MHC haplotype is from AS2 or BN, respectively.

Primary EAO Cultures. Testes were removed via the peritoneal cavity, decapsulated, and thoroughly minced with scissors. The fragments were agitated in cold phosphate-buffered saline (PBS) in trypsin bottles for 5 min to remove most of the intertubular cells. The tubular segments were softened by a 10-min incubation in Dulbecco's trypsin solution (Ca^{++} and Mg^{++} free, 0.25% trypsin) and then dissociated to single cells by further agitation in trypsin for 20 min. The single cell suspensions were resuspended in culture medium (Eagle's medium plus 15% heat-inactivated horse serum). The lymphoid cells were derived from peripheral and mesenteric lymph nodes by grinding the organs in loosely fitting tissue homogenizers. The lymphocytes were adjusted to a final concentration of 10×10^6 viable cells/ml culture medium.

For primary sensitization, 3 ml of the testis suspension was plated together with the same volume of lymphocyte suspensions into 60-mm surface-treated Petri dishes (Greiner, Nürtingen, W. Germany). On days 3 and 4 of the culture, 2 ml of the exhausted medium was removed and replaced by 3 ml of fresh culture medium. The cultures were harvested on day 5. To generate concanavalin A (Con A)-blasts, 30×10^6 lymph node cells were plated in a 5-ml culture medium into 60-mm dishes. Con A was added at a dosage of 50 μg/plate. The cultures were harvested after a 72-h incubation.

Density Gradient Centrifugation. The sensitized primary cell populations are composed of stimulated lymphoblasts, residual testis cells, small lymphocytes, and cell debris. Since all of these components are characterized by different buoyant densities, the lymphoblasts can be isolated by centrifugation in discontinuous Ficoll gradients. The harvested cell cultures were resuspended in 1 ml of *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid-buffered Eagle's medium (EM-H) and mixed with 6 ml of Ficoll stock solution (12.8 g Ficoll from Pharmacia, Freiburg, West Germany, dissolved in 30 ml EM-H). The mixture was placed on the bottom of a plastic tube fitting into the Beckman SW27 head (Beckman Instruments, Inc., Fullerton, Calif.). Sequentially, equal volumes (6 ml) of Ficoll-EM-H solutions, with decreasing densities ranging from 1.08 g/ml to 1.05 g/ml, with volumes of 6 ml each, were pipetted into the gradient that was finally topped by 4 ml of EM-H. The gradients were centrifuged in a Beckman ultracentrifuge, SW27 head, for 60 min, 10,000 rpm, at 4°C. The lymphoblast fractions contained >90% blast cells. This separation procedure did not affect cell viability as indicated by trypan blue dye exclusion tests.

The Regression Period. To allow the stimulated lymphoblasts to revert back to small secondary lymphocytes, they were resuspended in culture medium (3×10^5 cells/ml). 5-ml volumes of lymphoblast suspension, with 4 ml of allogeneic fibroblast suspension (embryonic rat fibroblasts, third to eighth passage; 1×10^5 cells/ml) and 4 ml of cell-free culture medium were plated into 100-mm surface-treated Petri dishes. In these cultures, most of the blast cells had reverted to small lymphocytes within 3 days. We collected the secondary lymphocytes several times, starting from day 3. As a result of one to two rounds of lymphoblast replication in these cultures, the recovery of secondary cells was always between 150 and 200% of the lymphoblast input.

Cryopreservation of Secondary EAO Cells. Secondary EAO cells collected from regression cultures were resuspended in 1.5 ml of culture medium. The same amount of culture medium containing 20% dimethyl-sulphoxide (DMSO) was slowly added at ice temperature. The mixtures were frozen and stored in styropore containers at −80°C. Shortly before use of the cells, the frozen samples were thawed under agitation in a 37°C water bath and washed in 40 ml of culture medium.

The Secondary Cultures. 100-μl volumes of resuspended secondary lymphocytes (2×10^6 cells/ml) were pipetted into the wells of microtiter plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). To these cultures, we added the same volumes of stimulator cells: either irradiated lymphocytes (2,000 R; 10×10^6 cells/ml), or trypsin-dissociated testis cells (2×10^6 cells/ml), unless otherwise stated. After 24 h, 20 μl of [^{3}H]thymidine solution (50 μCi/ml [^{3}H]thymidine; 0.5 μmol cold thymidine in EM-H) was added. After overnight incubation, the labeled cultures were harvested in a Titertek multiple harvester (Flow Laboratories, Irvine, Scotland) and the radioactivity was determined in a liquid scintillation counter.

Separation of T- and non-T-Lymphoid Populations. We applied a modified version of the technique of Parish and Hayward (15). Sheep erythrocytes (SRBC) were suspended in PBS at a concentration of 10%. To a 5-ml volume of 10% SRBC, we added 40 μl of a calibrated rabbit anti-SRBC antiserum (final dilution 1:125). The antibody-treated SRBC were washed twice and reconcentrated after a 20-min incubation at 37°C to 5%, and 5 ml of a 1:10 dilution of fresh mouse serum was added. After another 20-min period, the antibody plus complement-treated erythrocytes (EAC′) were washed twice in PBS and concentrated to 20%. 2.5 ml of lymphocyte suspension (4×10^7 cells/ml of Eagle's medium + fetal calf serum [FCS]) was mixed with an equal volume of EAC′ (20%). After a 15-min incubation in a roller tube at 37°C, the suspension was pipetted over a Ficoll-Hypaque layer and centrifuged in a siliconized glass tube at 3,000 *g* for 30 min at 20°C. The erythrocytes of the sediment fraction were lysed by KCl treatment at ice temperature. The nonrosetting lymphocytes contained virtually all the cells responsive to Con A, and <5% lymphocytes expressing membrane IgM, as detected by immunofluorescence. The rosetting fraction contained >80% Ig^+ cells.

Alloantisera. The procedure of alloantisera induction has been described elsewhere (16). Rats were primed with allogeneic thymus and spleen cells suspended in Freund's complete adjuvant (FCA). 0.5-ml volumes of FCA were injected into the foot pads and into multiple subcutaneous sites. After 3 wk, the animals were boostered by i.p. injections of lymphocytes suspended in PBS. Booster injections were repeated two to four times in biweekly intervals. For anti-MHC antisera, MHC-congenic L.BN rats were immunized with Lewis lymphocytes, and for non-MHC alloantisera, we injected BN recipients with MHC-identical L.BN cells. The antibody titers were monitored by the ^{125}I-protein A method as described by Dorval et al. (17).

Results

Specific Restimulation of in Vitro-Autoimmunized Memory T Cells. When lymphocytes from young adult rats are cultured with trypsin-dissociated testis cells from the same animal, a large number of lymphoblasts appears within 4–5 days. These blasts are able to induce progressive autoimmune orchitis when injected in vivo (9), and they can specifically destroy syngeneic testis cell monolayers in in vitro primary reactions (work in preparation). This autoimmune response can only be effected by lymphocyte populations containing immunocompetent T lymphocytes. T-cell deprived populations are incapable of forming autoimmune blasts. We isolated the autoimmune blasts from primary cultures by Ficoll density gradient centrifugation. The blasts were reincubated with allogeneic fibroblasts, which served as feeder cells. Within 3 days, nearly all the lymphoblasts in these regression cultures morphologically reverted back to small or medium-sized lymphocytes, which were collected repeatedly, stored at −80°C, and tested for their immune reactivity (Table I). These secondary EAO cells do not express IgM on their surface as was shown using an anti-IgM/^{125}I-protein A sandwich assay (data not presented).

TABLE I
Schematic Presentation of the Secondary EAO System

Step of the reaction	Day of culture	Cells involved	Cell number (lymphocytes)
Primary sensitization	0–5	Normal rat (T) lymphocytes + testis cells	200–300 × 10^6
Separation of blast cells		EAO-blasts	10–20 × 10^6
Regression phase	5–15	EAO-blasts + fibroblast feeders	
Harvest of secondary cells		Secondary EAO cells	10–30 × 10^6
Secondary sensitization	+36 h	Secondary EAO cells + fresh stimulators	–

TABLE II
Secondary Responsiveness of Secondary EAO Cells Derived from T-Cell Populations

Origin of responders*	Secondary stimulator testis cells		
	None	Lewis	AS2
Lewis lymph node cells	705 ± 68	4,090 ± 922	1,204 ± 135
Lewis spleen T cells‡	814 ± 121	16,133 ± 1,539	2,339 ± 641

* Fresh lymphocytes were autosensitized against autologous testis cells for 5 days. The blasts were isolated, transferred to allogeneic fibroblasts, and reverted to secondary EAO small lymphocytes. They were harvested and restimulated after 5 days.

‡ Fresh spleen cells were separated into T and non-T fractions by rosetting the cells with EAC′, and centrifuging the mixtures in Ficoll-Hypaque gradients.

Table II documents that Lewis secondary EAO lymphocytes derived from autosensitized lymph node cells strongly responded to syngeneic Lewis testis cells, but showed only marginal responsiveness when exposed to allogeneic AS2 testis cells. The same pattern of reactivity held true for secondary EAO cells derived from purified splenic T-cell populations, confirming that the reaction is indeed a T-cell response. In experimental groups controlling the antigenicity of the stimulator cells, fresh Lewis lymphocytes, as expected, responded to a higher degree to allogeneic stimulators than to syngeneic testis cells.

The stimulator cell specificity of the secondary reaction suggested, but did not prove, that immune recognition was the basis of the reaction. In contrast to secondary EAO cells which were supposedly selected for their immune responsiveness to self antigens, secondary lymphocytes derived from polyclonal Con A-activated T lymphoblasts should not be selected for antigen responsiveness. They should therefore exhibit a secondary response pattern similar to normal unprimed lymphocyte populations. To test this prediction, we stimulated Lewis lymph node cells with the polyclonal T-cell activator Con A, and purified the resulting blast cells by density gradient centrifugation. After removing attached Con A molecules by treatment with the hapten sugar α-methyl-mannoside, the blasts were allowed to revert back to small secondary lymphocytes on syngeneic fibroblasts.

TABLE III
In Vitro Restimulation of Secondary Con A Lymphocytes

Responder cells*	Con A‡	Stimulator lymphocytes		
		None	Lewis	AS2
Fresh Lewis	∅§	667 ± 98	1,084 ± 161	1,852 ± 228
	+		85,121 ± 5,573	83,340 ± 9,367
Secondary Con A (Lewis)	∅	4,943 ± 444	5,448 ± 1,414	8,430 ± 1,113
	+		88,954 ± 8,591	92,121 ± 11,644

* Freshly isolated lymph node lymphocytes; lymphocytes cultured for 72 h in the presence of 50 μg/ml Con A, before isolation and reversion to secondary cells.
‡ Final concentration of Con A in (+) cultures: 50 μg/ml.
§ ∅, Con A lacking from cultures.

The result of the restimulation experiments are presented in Table III. Syngeneic lymphocytes did not activate the secondary Con A cells, whereas a slight though definite activation was reached within a 36-h period in cultures containing allogeneic stimulator cells. The same pattern was obtained using fresh lymphocytes as responders.

Isolation of lymphoblasts was found to be an essential step for obtaining highly specific secondary EAO cells. By this procedure, at least theoretically, only such lymphoid cells are selected and allowed to revert back to small secondary EAO lymphocytes, which had been triggered in the primary EAO reaction. The small lymphocytes still present in the primary EAO cultures which presumably contain mainly the irrelevant, noncommitted clones, are thus eliminated.

To test secondary EAO cells for the presence of residual alloreactive clones, Lewis EAO blasts were incubated with allogeneic AS2 embryonic fibroblasts feeders. These regression cultures were maintained for 5 days. This period is sufficient for complete reversion of the lymphoblasts on the one hand, and, on the other, is known to allow competent alloreactive T lymphocytes to be sensitized against fibroblast alloantigens (18). If the transferred blasts were contaminated with lymphocyte clones responsive for alloantigens, these clones should have been activated by the alloantigens expressed on the AS2 fibroblasts. Consequently, in the secondary cultures, AS2 target cells should be more effective in reactivating the dormant EAO cells than irrelevant third party BN strain stimulator cells. As shown in Table IV, this was not the case. As expected, Lewis secondary EAO cells were specifically restimulated by the primary autoantigen, i.e. syngeneic Lewis testis or lymphoid cells. AS2 stimulator cells, which were MHC-identical with the feeder fibroblasts, were as uneffective as were the unrelated BN stimulators. A complementary experiment, using AS2 secondary EAO cells as responders and Lewis fibroblasts as feeders, showed the same pattern of responsiveness.

The Nature of the Stimulator Cells. As shown before in Table IV, only syngeneic, and not allogeneic, stimulator cells can restimulate secondary EAO cells. This is compatible with the notion that the secondary EAO cells were indeed primed against testicular autoantigens in the primary EAO cultures. The finding that not only syngeneic testis cells, but also lymphoid cells could

TABLE IV
Definitive Commitment of Secondary EAO Cells

Responder cells* (feeder fibroblasts)	Strain	Stimulator cells		
		None	Testis	Lymphocytes
Lewis (secondary EAO)	Lewis	763 ± 69	4,069 ± 798	27,410 ± 372
(AS2)	AS2		1,514 ± 193	3,033 ± 271
	BN		1,206 ± 193	4,761 ± 409
AS2 (Secondary EAO)	Lewis	550 ± 163	1,244 ± 120	3,203 ± 194
(Lewis)	AS2		3,419 ± 408	10,181 ± 1,259
	BN		744 ± 141	4,525 ± 388

* EAO blasts were cultured for 5 days on either AS2 or Lewis embryonic fibroblast monolayers before being tested for their antigen specificity.

TABLE V
Tissue Specificity of Secondary EAO Cells

Responder cells*	Strain	Stimulator cells		
		None	Testis	Fibroblasts‡
AS2 (secondary EAO)	AS2	307 ± 427	8,773 ± 738	−233 ± 344
	Lewis		978 ± 424	857 ± 564
Fresh AS2	AS2	143 ± 97	207 ± 52	89 ± 655
	Lewis		1,541 ± 731	1,589 ± 337

* Generation of the secondary EAO cells as in the other tables.
‡ cpm in cultures containing secondary EAO cells plus embryonic fibroblasts, minus fibroblast cultures.

re-elicit the reaction, however, was unexpected. It was in apparent contrast to the tissue-specific reaction pattern of primary EAO effector cells, which were found to cytostatically inhibit syngeneic testis monolayer forming cells, but not syngeneic embryonic fibroblasts (work in preparation).

Secondary EAO cells of the AS2 genotype were exposed to syngeneic testis and embryonic fibroblasts, and to stimulator cells of the allogeneic Lewis strain. Syngeneic fibroblasts were not capable of triggering the secondary EAO cells, although they were fully effective in primarily stimulating allogeneic fresh Lewis lymphocytes (Table V). Hence, fibroblasts do possess surface antigens recognizable by foreign T lymphocytes, but these antigens are not sufficient to stimulate secondary EAO cells as well.

The dose-response relationship of relevant syngeneic testis and lymphocyte stimulators (Fig. 1) demonstrated that a large excess of stimulator cells is required to reach maximal stimulation of the secondary EAO responder cells. In microwell cultures containing 1×10^5 Lewis secondary EAO responders, a plateau of the secondary response was only reached with 5×10^5 testis cells or 10×10^5 lymphocyte stimulators. This excess of stimulators could either mean that one responder cell requires several stimulators to be triggered, or that the stimulation capacity is restricted to a minor fraction within the stimulator population. Cell separation experiments suggested that this latter possibility is more likely.

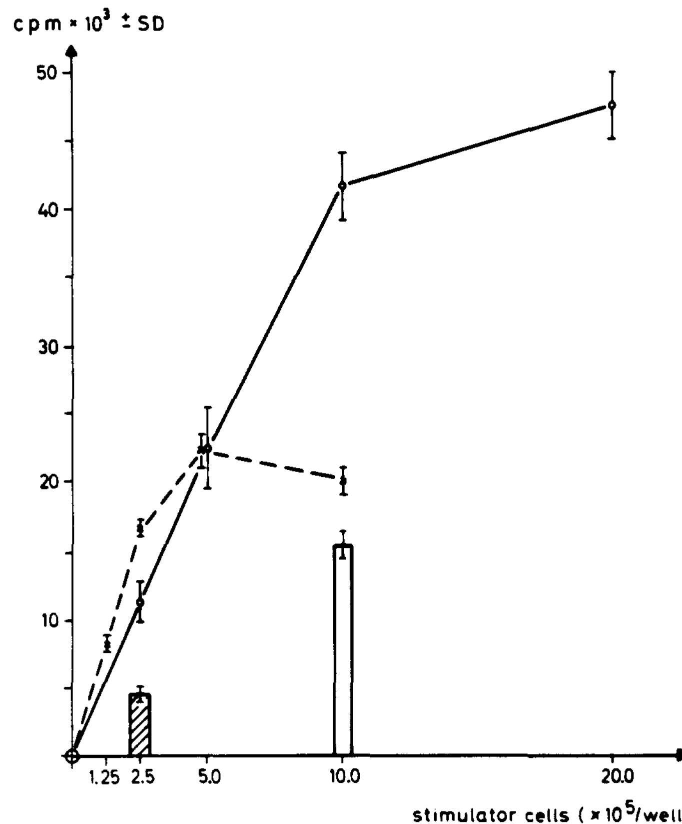

FIG. 1. Dose response of secondary EAO cells (Lewis) tested against increasing doses of syngeneic testis (×) and lymph node cells (O). Bars indicate stimulation by allogeneic AS2 testis (hatched) and lymph node cells (open). The [^{3}H]thymidine incorporation values are expressed as counts per minute ± standard deviations.

TABLE VI
Buoyant Density of the Testicular Stimulator Cell

Responder cells*	Separated testis stimulator cells‡				
	None	A'	A	B	C/D
Secondary EAO (Lewis)	501 ± 431	4,944 ± 583	12,261 ± 627	13,657 ± 2,298	24,753 ± 2,879
None	–	142 ± 76	451 ± 171	2,533 ± 195	1,152 ± 358
Cell distribution, %		1.04	1.05	1.06	1.07–1.08
Buoyant density, *g/ml*		Germinal epithelium ----------------			
Major cell component			----------------------------Sertoli-like cells----------------------------		

* Generation of secondary EAO cells as in the other tables. The cells were frozen and stored in the presence of 10% DMSO, at −80°C for 3 wk.

‡ Fractionation of freshly trypsin-dissociated testis suspensions in Ficoll gradient. All the wells contained 2 × 10⁵ testis cells derived from the single bands.

Enzyme-dissociated testis cell populations are composed of various distinct single cell populations (19, 20). Centrifugation in isopycnic Ficol density gradients allows semiquantitative separation of germinal epithelium cells from somatic cells, such as Sertoli and Leydig cells (21). We incubated Lewis secondary EAO cells with constant doses of syngeneic testis cell fractions separated by density gradient centrifugation. Table VI shows that germinal cells, which

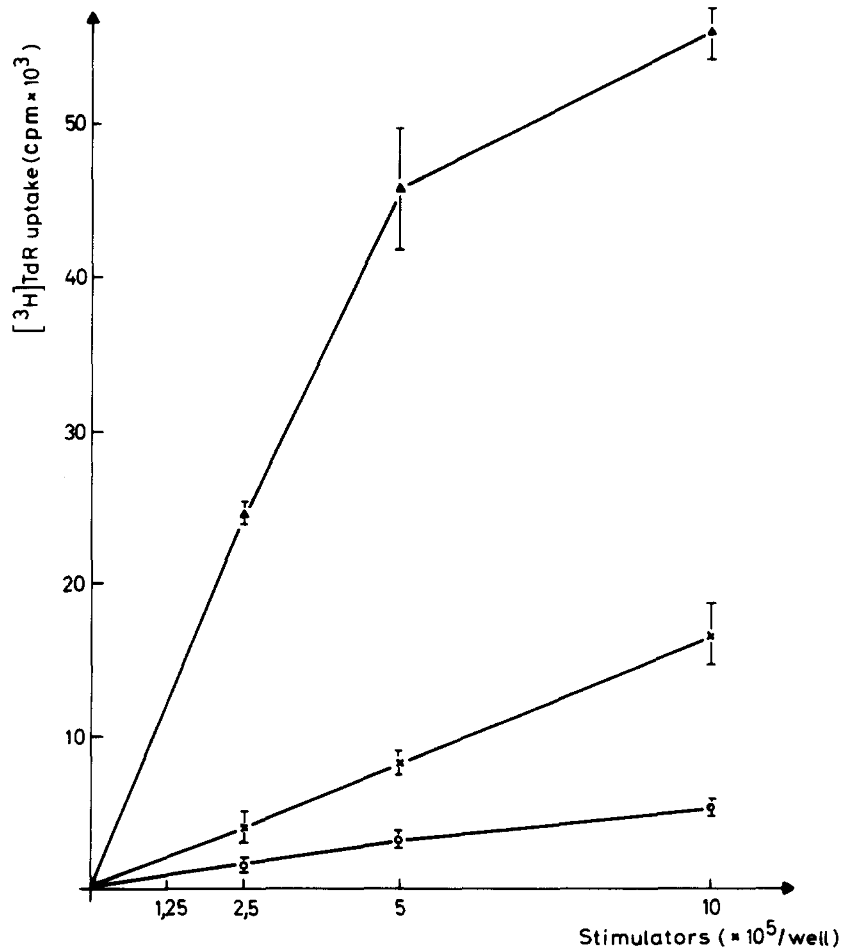

FIG. 2. Dose response of secondary EAO cells (Lewis) tested against increasing doses of syngeneic thymus cells (×), lymph node T cells (O), and lymph node non-T cells (——). The stimulation values are expressed as in Fig. 1.

constitute the majority of all testis cells, are inefficient in restimulating secondary EAO cells. There was a direct relation between the capacity of testis cell fractions to elicit secondary EAO responses, and their relative content of Sertoli cells. This, together with our previous finding that self-responsive lymphocytes form rosette-like aggregates around Sertoli cells (9), suggests (*a*) that only a subpopulation within the testis cells is the relevant autoimmune stimulator, and (*b*) that this cell population is probably identical with Sertoli cells.

Also in the case of lymphoid cells, only a minor subpopulation can stimulate the secondary EAO cells. These cells do not occur in T-cell populations which were selected for their lack of Fc and complement receptors. The effective stimulators were, however, enriched in Fc and complement receptor-positive fractions (Fig. 2). The observation that the stimulator cells are adherent to glass surfaces, and that unseparated thymocyte suspension which is known to contain very few Ig^+ B lymphocytes, does contain a significant number of

TABLE VII
MHC Specificity of Secondary EAO Cells

Responder cells*	Strain	Stimulator cells		
		None	Testis	Lymphocytes
Secondary Lewis (secondary EAO)	Lewis	67 ± 21	3,116 ± 420	20,665 ± 1,778
	L.AS2		363 ± 105	2,753 ± 181
	AS2		458 ± 78	2,092 ± 137
Fresh Lewis	Lewis	209 ± 26	470 ± 117	406 ± 187
	L.AS2		681 ± 67	1,666 ± 256
	AS2		707 ± 78	1,404 ± 79
L.AS2 (secondary EAO)	Lewis	277 ± 138	870 ± 104	3,656 ± 380
	L.AS2		10,157 ± 764	40,374 ± 2,894
Fresh L.AS2	Lewis	175 ± 49	518 ± 57	987 ± 33
	L.AS2		371 ± 30	181 ± 43
AS2 (secondary EAO)	Lewis	307 ± 427	978 ± 424	15,494 ± 457
	L.AS2		8,567 ± 986	23,789 ± 502
	AS2		8,773 ± 738	31,946 ± 1,568
Fresh AS2	Lewis	143 ± 97	1,541 ± 731	1,367 ± 76
	L.AS2		310 ± 210	149 ± 23
	AS2		207 ± 52	116 ± 28

* As in Table VI.

macrophages, suggests that the stimulator cell within the lymphoid populations is a macrophage-like cell.

The Role of the MHC. Strong histocompatibility antigens play a critical role in the secondary EAO response. This was found in testing secondary EAO responder cells of various genetic makeup for their specificity by confronting them with stimulator cells of a suitable genotype (Table VII). Lewis secondary EAO cells vigorously respond to Lewis testis and lymph node cells, but react to a lesser degree against either allogeneic AS2 or MHC-congenic L.AS2 stimulator cells. Conversely, AS2 secondary EAO cells can be optimally triggered by both AS2 and L.AS2 stimulators, but they are triggered less by MHC-incompatible Lewis testis and lymphoid cells. These data, along with the observation that L.AS2 secondary EAO cells do not respond to Lewis stimulators which are genetically identical with L.AS2 except of the MHC, emphasize the crucial role of MHC determinants in the EAO response.

These results are complemented by serological experiments. We tested the effect of either anti-MHC, or anti non-MHC alloantisera, on the stimulatory capacity of syngeneic stimulator cells. Since it is known that anti-MHC antibodies strongly interfere with antigen recognition by T lymphocytes (16), we pretreated stimulator cells with anti-MHC alloantisera before testing their capacity to stimulate either Lewis secondary EAO cells or fresh Lewis lymph node cells. Anti-MHC sera significantly reduced the stimulatory capacity of syngeneic stimulator cells. The lack of total inhibition could be the result of partial reappearance of MHC antigens during the secondary culture. Anti-

TABLE VIII

Effect of Antiserum Pretreatment on Secondary Response

Responder Cells*	Antiserum‡	Stimulator cells		
		None	Lewis-LN	AS2-LN
Secondry EAO (Lewis)	∅§	1,267 ± 347	18,453 ± 297	3,010 ± 324
	α-H1	–	6,459 ± 761	2,852 ± 146
Fresh Lewis LN	∅	601 ± 86	656 ± 167	3,380 ± 334
	α-H1	–	986 ± 152	2,582 ± 280
Secondary EAO (Lewis)	∅	1,143 ± 291	27,776 ± 637	5,422 ± 981
	α-BG	–	34,006 ± 2,491	5,532 ± 564
Fresh Lewis LN‖	∅	2,805 ± 522	2,805 ± 522	20,023 ± 1,884
	α-BG	–	3,928 ± 328	16,712 ± 1,644

* As in Table VI.
‡ α-H1, L.BN-anti-Lewis antiserum directed against MHC determinants exclusively; α-BG, BN-anti-L.BN antiserum directed against Lewis-background.
§ ∅, lacking antiserum.
‖ These cultures were labeled and harvested 24 h later than the other groups.

TABLE IX

Effect of Heterologous and Autologous Serum on Secondary EAO Response

Responders*	Stimulators‡	Serum additives§			
		Horse serum	Horse serum/rat serum (1:1)	Rat serum	No serum
Lewis (secondary EAO)	Lewis-LN	33,201 ± 2,248	13,141 ± 1,390	6,443 ± 153	11,914 ± 2,074
	AS2-LN	4,025 ± 685			
	Lewis-testis	21,151 ± 744	11,501 ± 1,851	4,882 ± 301	11,433 ± 749
	AS2-testis	4,228 ± 632			

* As in Table VI.
‡ Lewis or AS2 lymph node cells, irradiated 2,000 R; testis cells, trypsin-dissociated.
§ Final serum concentration: 15%; horse serum: 7.5% horse serum, and 7.5% autologous rat serum, heat-inactivated.

Lewis alloantisera directed against non-MHC alloantigens exclusively, did not interfere with the anamnestic autoimmune response (Table VIII).

Culture Medium Factors Do Determine the Secondary EAO Response. The data suggest that MHC antigens determine the specificity of the secondary EAO response, and that the stimulatory capacity is restricted to certain cell types. It is, however, not clear whether these autoantigens are recognized in a native state as unaltered, genuine autoantigens, or rather as self structures which are modified by foreign determinants. In both the primary EAO cultures and the subsequent culture steps, foreign reagents such as horse serum and 2-mercaptoethanol (2-ME) are present. At least in the case of heterologous sera, it has been repeatedly stressed that some components readily bind to cultured cells (22, 23).

Secondary EAO cells which had been generated in a culture medium supplemented with horse serum, were tested for their responsiveness to syngeneic targets in the presence of horse serum, in the absence of any serum, in the presence of autologous rat serum, and in culture medium containing a 1:1 mixture of horse and rat serum. Table IX shows that the secondary EAO

TABLE X
Independence of Secondary Stimulation of Horse Serum and 2-ME Culture Medium Additives

Medium additives*	Stimulator lymphocytes‡		
	None	Lewis	AS2
Horse serum + 2-ME	418 ± 32	19,601 ± 3,868	9,014 ± 627
Horse serum ∅§ 2-ME	609 ± 112	17,134 ± 1,547	5,757 ± 290
∅ Horse serum + 2-ME	314 ± 68	8,643 ± 1,116	3,322 ± 367
∅ Horse serum ∅ 2-ME	212 ± 84	5,084 ± 783	2,305 ± 364

* HS: 15% horse serum, heat-inactivated; 2-ME: 0.05 mM 2-mercaptoethanol; responder cells: secondary EAO cells, Lewis strain, generated as in Table VI.

‡ Fresh, irradiated lymph node cells (2,000 R); counts per minute ± standard deviation.

§ ∅, absence of additive.

response did not depend upon horse serum determinants. A significant reaction response was triggered in the complete absence of horse serum. Rat serum, on the other hand, strongly depressed the secondary EAO response. This was the case even when it was mixed with horse serum.

2-ME is the other agent which could possibly act as a self modifier. To determine the role of 2-ME in the EAO reaction, secondary EAO cells which were grown and maintained in medium containing both horse serum and 2-ME (but no antibiotics or other foreign materials), were exposed to syngeneic and allogeneic stimulator lymphocytes in the presence or absence of both reagents. Horse serum, and to a lesser degree, 2-ME, are required to obtain a maximal secondary response. In culture lacking both reagents, the amplitude of the response is lowered, but more important, the degree of specificity of the response is unaffected (Table X).

Discussion

T lymphoblasts which have been activated in mixed cultures against autologous testis cells, will revert back to small, functionally and morphologically quiescent lymphocytes after further incubation in the absence of testicular tissue. When these secondary lymphocytes (secondary EAO cells) are reexposed to syngeneic testis or lymphoid cells, they respond by vigorous transformation and proliferation.

The secondary proliferation response could thus constitute a memory autoimmune response. Alternatively, freshly added syngeneic stimulator cells might act as inert filler cells (24), or as collaborator cells required by the secondary lymphocytes to react against nonspecific mitogenic stimuli (25, 26).

The observation that secondary lymphocytes derived from polyclonal Con A blast cells do not preferentially proliferate when cultured together with fresh syngeneic specificity, argues against this interpretation. Moreover, the secondary reactions are not only characterized by cell proliferation, but also by the generation of effector T cells which specifically destroy syngeneic testis target cells (work in preparation). These points suggest that the secondary EAO

reaction is indeed a secondary immune reaction of memory cells which were primed in vitro against autologous testis antigens.

Our data suggest that in the secondary EAO reaction, the recognition of MHC determinants plays an essential role. This has been shown by confronting secondary EAO cells with stimulator cells which are either syngeneic, allogeneic, or congenic with respect to the MHC. Only those stimulator cells which shared the MHC with the autosensitized responder cells could elicit a full-scale secondary response. Minor transplantation antigens did not play demonstrable roles. This was confirmed in experiments where syngeneic stimulator cells were pretreated either with antibodies directed against MHC determinants exclusively, or against non-MHC determinants. Although both types of antibodies bound to the stimulators in comparable quantities, only the anti-MHC antibodies decreased the efficiency of the stimulator cells.

The degree of MHC specificity of the secondary EAO reaction is high. Proliferation responses against the relevant autoantigens, in most but not in all cases, are about 10-fold greater than those against irrelevant allogeneic stimulator cells, and thus compare favorably to mouse secondary cells primed in allogeneic mixed lymphocyte cultures (27). The finding that secondary EAO cells, in contrast to polyclonal fresh lymph node cells, cannot be sensitized against allogeneic fibroblasts, argues against the possibility that unspecific clones contaminating the secondary cell populations are responsible for the cross-reactions. As in T-cell responses against virus- or tumor-modified self (28, 29), it appears that in the primary EAO reaction, various clones of T cells are being triggered, and that some of those may be capable of cross-reacting with determinants on non-self cells.

Recognition of MHC self-antigens is known to play a critical role in a number of T-lymphocyte responses. These include immune responses against virus-infected target cells (1), against chemically modified cells (2), against tumor cells (30, 31), against target cells bearing foreign minor transplantation antigens (32–34), in delayed hypersensitivity (35), and possibly even against conventional soluble antigen presented on macrophages (3, 36). Consequently, the MHC determinants recognized by the secondary EAO cells in our system could be recognized in a native, unaltered state, or alternatively, in conjunction with foreign agents, which were introduced by the tissue culture conditions. As previously stated (14), there are indeed multiple possibilities in our tissue culture systems, which theoretically could lead to an artificial alteration of cell surface antigens. Thus, we are using trypsin-dissociated testis cells as priming antigens. Protease treatment could either expose normally hidden antigens, or alternatively, mutilate exposed structures, thus altering their antigenicity (37, 38). This possibility seems to be improbable, since secondary EAO cells can be reactivated not only by trypsinized testis cells, but also by testis cells, which were dissociated mechanically, and, additionally, by untreated lymphoid cells.

Heterologous serum factors whose role in in vitro models of autoimmune induction has been repeatedly stressed (22, 23), do not play demonstrable roles in determining the specificity of the secondary EAO cells. As already reported, lymphocytes autosensitized in horse serum display identical specificity patterns when challenged in secondary cultures in the presence of either horse serum,

or non-cross-reacting FCS additives (14).

We demonstrated that secondary EAO cells primed in the presence of heterologous sera also specifically respond in secondary cultures in the total absence of serum. Autologous serum, which actively suppresses primary autosensitization (10, 39), also effectively interferes with the secondary proliferative response. It is not yet clear whether this effect is the result of specific immunosuppressive factors, or of unspecific immunosuppression or toxicity. The other foreign additive contained in our cultures, 2-ME, was also shown not to influence the specificity of the secondary EAO reaction. These findings, together with the fact that the secondary EAO cells can be restimulated only by very few, distinct cell types (*vide infra*), and not by all cells bearing syngeneic MHC antigens on their surface, make it improbable that the secondary EAO response is directed against MHC autoantigens altered by foreign tissue culture constituents.

A most intriguing possibility of self modification is in the neoexpression of endogenous virus products on the cell membrane. Such neoantigens have been demonstrated to appear spontaneously (40), after treatment of lymphocytes with either irradiation or 5-bromo-2′-deoxyuridine (41), with B-cell activators (40), or in the course of mixed lymphocyte culture or graft-vs.-host reaction (42). In all these instances, expression of demonstrable virus products required a minimum latent culture period of 24 h. In our secondary EAO cultures, self recognition and response was manifested as early as 6–12 h of culture, expressed by formation of characteristic mixed stimulator-responder cell aggregates. This short period and the tissue specificity of the stimulator cells (*vide infra*), which would necessitate a tissue-restricted expression of the endogenous viruses, make this explanation less probable. Further experiments are required to clarify this issue.

Our results suggest on one hand that major transplantation antigens determine the specificity of the secondary EAO response. On the other hand, the capacity to stimulate secondary EAO cells is restricted to a few distinct cell populations. This is suggested first by the observation that only testis and lymphoid cell populations can restimulate secondary EAO cells. Syngeneic fibroblasts, which readily elicit a primary response by fresh lymphocytes, however, do not stimulate secondary EAO cells. Secondly, we found that among the relevant testis and lymphoid populations, only minor subpopulations are the active stimulators, whereas the majority of all cells is inactive.

In testis populations, these active stimulators can be enriched by centrifugation in discontinuous density gradients. The separated testis fractions with the highest EAO-stimulating capacity contain the highest proportion of cells which not only resemble Sertoli cells in fresh smears (43, 44), but in addition, give rise to fast-growing monolayers, which also meet the morphological criteria of Sertoli cell-derived monolayers (45, 46). Since the same cell type has previously been found to bind preferentially those lymphocyte clones which are reactive against in the EAO reaction (9), it appears reasonable to assume that the EAO response is directed against autoantigens expressed on these Sertoli-like cells.

In lymphoid stimulator populations, the active stimulator cells are present in cell fractions possessing Fc and complement receptors. These stimulators

seem to adhere to glass beads and thus behave like macrophages.

Similar cells may be responsible for primary sensitization of T lymphocytes by syngeneic non-T lymphocytes, which has been reported repeatedly (47–50). Experiments carried out by our own group as well as by others (51), indicate that specifically self-reactive memory cells can be generated in mixed syngeneic T/B-lymphocyte cultures. Furthermore, we found that secondary lymphocytes which were primed against syngeneic non-T cells, can be restimulated by syngeneic non-T cells as well as by testicular Sertoli cells (unpublished results). The symmetric cross-reactivity between autoantigenic Sertoli cells and macrophage-like cells is remarkable, since both cell types share important functional properties. Both are phagocytes playing roles in removal of undesirable autologous tissues, such as residual bodies that are removed by Sertoli cells during late stages of spermiogenesis (52).

Tissue, as well as MHC specificity of the secondary EAO response, could be explained in two ways. Several groups reported that minor transplantation antigens (32), or H-Y antigens (33), are recognized by MHC-compatible T lymphocytes and determinants of the murine H-2 system. It could be that in the EAO response, tissue-specific autoantigens, which are not coded for by the MHC, are also recognized by the responsive T cells in conjunction with MHC self antigens.

Alternatively, we cannot rule out the possibility that the EAO reaction is directed against MHC determinants exclusively, and that these MHC determinants are expressed only on the active stimulator cell populations. MHC antigens with restricted tissue distribution are the Ia determinants of the murine H-2 system. It is noteworthy that some of the Ia antigens are expressed on macrophages, but not on T cells or fibroblasts. Although we know of no reports of their presence on Sertoli cells, the same Ia antigens have been demonstrated on sperm cells (53). During spermiogenesis, Sertoli cells establish firm contacts with the maturating sperm cells. They may thus present sperm Ia antigens, which could cross-react with macrophages. The question of whether or not sperm cells can elicit secondary EAO responses by themselves, is still open. They can, however, initiate MLC-like reactions in allogeneic sperm-lymphocyte combination cultures (54, 55).

Summary

Immunological memory has been induced in vitro against testicular autoantigens by priming normal rat T lymphocytes against autologous testis cells, and by permitting the isolated blast cells to revert back to small secondary lymphocytes (secondary EAO cells) in the absence of the priming antigen. The secondary EAO cells vigorously respond in a secondary response when reconfronted with syngeneic testis or lymphoid cells. Their responsiveness to nonself stimulator cells is, however, reduced. Secondary cells derived from concanavalin A-stimulated blasts, do not show that pattern of specificity. The specificity of the secondary EAO cells is definite, and cannot be affected by further culture on allogeneic fibroblasts, which are antigenic for unprimed T lympho-

cytes. At least part of the autoantigens are determined by the major histocompatibility gene complex (MHC). Factors provided by the culture system do not appear to determine the specificity of this reaction. Only minor cell populations can restimulate secondary EAO cells. One of these populations is presumably testicular Sertoli cells, the putative primary autoantigen. Moreover, macrophage-like cells within the lymphoid populations can elicit a secondary EAO response. Thus, the autoantigens relevant in the secondary EAO response are either MHC antigens restricted to these testicular and lymphoid subpopulations, or MHC antigens recognized in conjunction with organ-specific non-MHC determinants.

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