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THYMUS ORIGIN OF LYMPHOCYTES REACTING AND STIMULATING REACTION IN MIXED LYMPHOCYTE CULTURES—STUDIES IN THE RAT

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SUMMARY

Mixed lymphocyte cultures between pairs of allogeneic cell suspensions from rat thymus of one strain versus thymus, lymph node, spleen or bone marrow from a histo-incompatible strain, have shown a decreasing gradient of stimulation capacity. After complete neonatal thymectomy 6 weeks previously, lymph-node lymphocytes are shown to have diminished or absent capacity to stimulate allogeneic rat thymocytes in mixed culture. It it concluded that thymus-derived lymphocytes appear to be required both for stimulation as well as for reaction in the mixed lymphocyte test.

INTRODUCTION

Since the original description of blastic transformation in cultures of peripheral blood lymphocytes derived from genetically different human donors (Bain, Vas & Lowenstein, 1964), this reaction has been intensively investigated. It is now established that the mixed lymphocyte reaction (MLR) is a valid indicator of difference at one or more major histocompatibility loci between human lymphocyte donors (Amos & Bach, 1968; Schellekens *et al.*, 1970). Silvers, Wilson & Palm (1967) have presented evidence which strongly suggests that in the rat, response in the MLR is similarly dependent upon difference between responding inbred rat strains determined by the major histocompatibility locus AgB.

Schwarz (1967, 1968) has reported several studies of the MLR in rats, using thymus spleen or lymph nodes as the source of the participating lymphocytes, but without assessment of reactivity between lymphocytes of different tissue origin. Wilson (1967), Wilson, Silvers & Nowell (1967) and Wilson, Blyth & Nowell (1968) have described similar and additional experiments, with extensive assessments of the optimal conditions necessary for rat lymphocyte culture, and evidence that lymphocytes responding in the MLR are thymus derived.

There is evidence in mice of antigenic difference between thymus derived and non-thymus-

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derived lymphocytes (Reif & Allen, 1964; Raff 1969) and Iversen (1970) has recently reported a similar conclusion from work on inbred rats using different techniques. It seemed possible that the extra antigen found upon the surface of thymus-derived lymphocytes might be relevant in the MLR, both for reaction to allogeneic cells and also for stimulation of this response.

The present report describes studies on the MLR in inbred rat strains using interstrain mixtures of thymocytes and thymocytes mixed with lymphocytes derived from different lymphoid organs. The evidence indicates a descending gradient of reactivity from thymus, to lymph node, to spleen, to bone marrow. Lymphocytes of mature animals previously subjected to neonatal thymectomy, show diminished or absent capacity to *stimulate* allogeneic thymocytes, thus suggesting a thymus origin for lymphocytes capable of stimulating as well as reacting in the mixed lymphocyte reaction.

METHODS

Inbred rats of the AS_2 and HS strains were used throughout. These strains are known to show histocompatibility differences determined by the major AgB histocompatibility locus (Heslop 1968). Lymphocyte suspensions were prepared from animals after complete exsanguination under ether anaesthesia, using the technique of needle aspiration of the abdominal aorta. Blood from both strains was pooled and the serum obtained was stored at $-20°C$.

The thymus, lymph nodes and spleen were removed aseptically, washed in Hanks' balanced salt solution to remove any adherent blood, and dissected free of fat and other tissue. The thymus and lymph nodes were separately minced with fine scissors in fresh Hanks' solution. The resulting cell suspensions were passed through fine nylon-gauze mesh and then centrifuged for 5 min at 1000 rev/min. After one further wash in Hanks' solution, cell viability was checked by the trypan blue method and the lymphocyte deposit was resuspended in culture medium at a concentration of 2×10^6 viable cells/ml. Cell suspensions from the spleen were prepared by gentle homogenization in Hanks' solution, using a simple pestel in a sterile glass tube. The cell suspension was then passed through nylon-gauze mesh and handled as for the thymus and lymph node preparations.

Bone marrow cell suspensions were obtained by washing out with Hanks' solution, the marrow space of the shaft of the femur on both sides. The bones were first dissected out asceptically. The marrow particles were gently homogenized as with the spleen and the resulting cell suspension was passed through two layers of nylon-gauze mesh prior to washing and resuspension in culture medium. Approximately one third of the marrow cells obtained in this way were small lymphocytes.

The culture medium used throughout was RPMI, supplemented with 10% foetal-calf serum and 10% pooled rat serum (used fresh or within 2–3 weeks of collection). For all experiments, white cell suspensions were cultured at a concentration of 2×10^6 per ml. They were grown in $3 \times \frac{1}{2}''$ capped glass tubes and were maintained for 4 days at 37°C in an atmosphere of 5% CO_2 95% air. Preliminary assessment had indicated that with these methods, response in mixed cultures was maximal at the 3rd and 4th day and fell off markedly by the sixth day of culture. Contrary to previous findings with medium 199, survival of unstimulated rat lymphocytes in RPMI supplemented as described, was found to be satisfactory at 4 days.

After preliminary morphological assessment of rat mixed lymphocyte cultures, all subsequent analysis of transformation potential was made on the basis of comparisons of tritiated thymidine incorporation into DNA for unmixed and mixed lymphocyte populations. All cultures received tritiated thymidine (0·5 μCi/ml—specific activity 150 mCi/mM) after 72 hr and were harvested 24 hr later. The method of processing and counting thymidine treated cultures was that described by Knight & Ling (1969).

Thymocytes from one strain were mixed in culture in equal numbers with lymphocytes from the other incompatible strain, these latter cells being derived either from thymus, lymph nodes, spleen or bone marrow. Control cultures of each lymphocyte suspension separately were set up for each experiment. All cultures were performed in triplicate and the results recorded are the means of triplicate counts. Syngeneic mixtures of lymphocytes of different tissue origin were also set up as additional controls for each experiment.

Where indicated in the text, neonatal thymectomy was performed on AS_2 animals within 24 hr of birth by a sternal split procedure under deep hypothermia, and application of suction for removal of the thymus. Histological assessment for completeness of removal was carried out at the time of killing. In sham thymectomized animals, the sternum was split under hypothermia but the thymus was left intact.

Rabbit anti-rat thymocyte serum was produced by five-weekly intravenous injections of 3×10^7 thymocytes. Serum was obtained 1 week later and after heat inactivation for 30 min at 56°C, it was repeatedly absorbed with washed rat red cells in the cold. The serum was administered to newborn AS_2 strain rats twice weekly by intraperitoneal injection in a dose of 1 ml for a total of seven injections. Lymphocyte suspensions were prepared from the treated animals 1 week later.

RESULTS

These are set out in Tables 1–3 and summarized in Fig. 1. In all experiments, maximal stimulation of ^3H tdr. incorporation was observed in mixed cultures of incompatible thymocytes, and least stimulation was found in cultures with bone-marrow lymphocytes. Thymocytes versus lymph node or spleen lymphocytes produced intermediate levels of stimulation. The same pattern and gradient of response was observed when cultures were made 'one way' by X-irradiation (1500 r) of the stimulating cell suspensions. As expected the stimulation factor after irradiation was more than halved for thymocyte-versus-thymocyte cultures but reduction was very much less in mixtures with other cell suspensions, possibly indicating that most of the increase in thymidine incorporation in the latter mixed cultures is a function of the thymocyte rather than the tissue lymphocytes; or that intact lymph node and spleen lymphocytes may have an inhibitory influence on thymocytes.

Interpretation of mixed cultures, with stimulation by lymphocytes of non-thymic origin was complicated by the fact that in autologous mixtures of tissue lymphocytes with thymocytes, mild but definite stimulations of ^3H tdr. incorporation was observed (Table 2). The net stimulation results shown in the Tables were obtained by subtraction of the autologous from the homologous stimulation values. Following X-irradiation of the stimulating cell suspensions, this non-allogeneic increased stimulation was largely eliminated but autologous mixtures then showed some reduction of observed as compared with expected counts, presumably as a consequence of cell death. Accordingly, it has still seemed desirable

TABLE 1. Stimulation of ^{3}H-thymidine incorporation by mixing thymocytes and allogeneic tissue lymphocytes

Two-way (without irradiation)					One-way—by irradiation—1500 r— of *stimulating* lymphocytes from thymus, lymph node, spleen or bone marrow				
Mean DPM cultured separately	DPM in mixed culture	*Net DPM in mixed culture	Stimulation index (S.I.)	Mean S.I.	Mean DPM cultured separately	DPM in mixed culture	*Net DPM in mixed culture	Stimulation index (S.I.)	Mean S.I.
Thymus-versus-thymus									
162	4250	—	26		57	557	—	9·7	
423	6311	—	15		17	128	—	7·5	8·0
320	4480	—	13·4		91	610	—	6·7	
170	8430	—	47						
399	5696	—	14·2	24					
250	4787	—	20						
236	4021	—	18						
77	2060	—	28						
75	2732	—	38						

Thymus-versus-lymph node			*Net S.I.*	*Mean Net S.I.*				† *Net S.I.*	*Mean Net S.I.*
30	174	156	5·2		14	95	106	7·6	
157	1057	939	5·1	5·2	51	140	162	3·2	6·9
113	870	714	6·3		37	335	300	8·1	
142	715	610	4·3		92	829	796	8·6	
Thymus-versus-spleen									
162	655	621	3·8		14	62	42	3·0	
40	185	110	2·7		46	52	87	1·9	3·8
96	301	163	1·8	3·1	37	124	135	6·6	
55	331	253	4·6						
125	358	338	2·7						
Thymus-versus-bone marrow									
159	331	245	1·5		52	72	124	2·4	
104	213	197	1·9	1·6	73	34	119	1·6	
107	458	171	1·5		20	50	49	2·4	1·6
					45	47	0	0	

* Net DPM = count obtained—DPM increment in the corresponding *autologous* mixed culture (see Table 2)

† Net S.I. = stimulation index. i.e. $\frac{\text{net DPM in mixed culture}}{\text{mean DPM from same lymphocytes cultured separately.}}$

to subtract the autologous from the homologous stimulation values obtained to correct for this.

The stimulation indices for one way and two way mixed lymphocyte reactions are compared in Fig. 1. The gradient in stimulation capacity of lymphocytes from various sources is well seen. The low response by thymocytes to intact or irradiated spleen and bone marrow lymphocytes might be explained in part as due to contaminating red cells, polymorphs etc., but this objection does not apply to the lymph-node cell suspensions which were virtually free of non-lymphocytic elements.

TABLE 2. Stimulation of ^{3}H-thymidine incorporation by mixing thymocytes and **autologous** tissue lymphocytes

Two-way (without irradiation)				One-way (after irradiation of lymph node, spleen or bone marrow lymphocytes)			
Mean DPM cultured separately	DPM in mixed culture	Stimulation index (S.I.)	Mean S.I.	Mean DPM cultured separately	DPM in mixed culture	Stimulation index (S.I)	Mean S.I.
Thymus-versus-lymph node							
9	12	1·3		42	20	0·5	
104	302	2·9		19	9	0·5	0·8
164	363	2·2	1·9	18	22	1·2	
100	138	1·4		52	54	1·0	
Thymus-versus-spleen							
122	133	1·1		45	10	0·2	
116	283	2·4		40	15	0·4	0·4
19	54	2·8	1·9	18	8	0·4	
60	125	2·1		49	34	0·7	
84	86	1·0					
Thymus-versus-bone marrow							
283	396	1·4		119	25	0·2	
83	123	1·5	2·1	70	14	0·2	0·6
153	538	3·5		51	55	1·1	
				13	14	1·1	

In an attempt to overcome this objection and to demonstrate in a different and more conclusive manner the role of thymus-derived lymphocytes in *stimulating* the mixed-lymphocyte reaction, experiments with anti-thymocyte serum treated animals and with neonatally-thymectomized animals were carried out.

The results of treatment with antithymocyte serum (ATS) proved rather inconclusive because the serum appeared to stimulate thymidine incorporation by lymphocytes from treated animals, independently of any allogeneic-lymphocyte stimulation. By subtraction of the values obtained with isogeneic-lymphocyte mixtures, it does appear that the stimulation

index for intact thymocytes, versus lymphocytes from ATS treated animals has been moderately reduced. The results of one such experiment are shown in Table 3.

The results after neonatal thymectomy provide more definite evidence that thymus-derived lymphocytes are required to *stimulate* the mixed lymphocyte reaction since, after total thymectomy lymph-node lymphocytes showed diminished or absent capacity to stimulate intact allogeneic thymocytes (Table 3). Lymph-node lymphocytes from the thymectomized animals were not obtained until 5 weeks or more after operation, and at this time immunological maturation, at least in respect of skin-graft rejection times, is

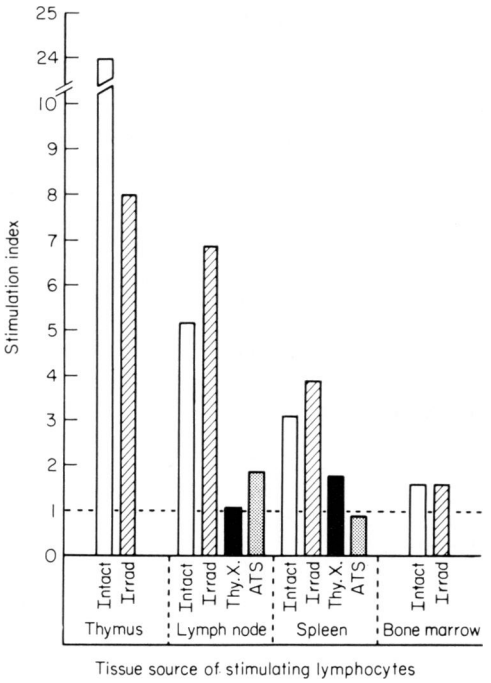

FIG. 1. Mean stimulation indexes: Mixed culture of normal thymocytes with allogeneic stimulating lymphocytes from thymus lymph node, spleen or bone marrow.

For all tissues except thymus, the stimulation index is net (see Tables 1 and 2). Irrad. = lymphocytes X-irradiated at dose of 1500 r. Thy. X. = lymphocytes from mature rats after neonatal thymectomy. ATS = lymphocytes from mature rat, treated with antithymocyte serum from birth.

known to be in the adult range (Heslop, 1969). If thymectomy was incomplete no depression of mixed lymphocyte response was observed.

When the MLR was made one way, by X-irradiation (1500 r) of the stimulating-lymphocytes derived from the complete neonatally-thymectomized group, partial restoration of stimulant capacity by these cells was observed in one experiment but not in a second experiment. No convincing explanation for this change in response can be given. It is similar in kind though different in magnitude to that observed with mixed cultures of thymocytes and X-irradiated lymph-node lymphocytes from intact animals.

DISCUSSION

The frequency with which mouse lymphocytes carrying the θ-thymus antigen marker can be detected in various lymphoid organs shows a gradient, with maximal numbers in thymus and thoracic duct (90–100%) and decreasing numbers in peripheral blood, lymph node, spleen and bone marrow in that order (Raff, 1969). A similar gradient has been demonstrated in rats by Iversen (1970), employing specific antisera against recirculating and non-recirculating lymphocytes.

TABLE 3. ^{3}HTdr Incorporation after stimulation of normal allogeneic thymocytes with lymphocytes (lymph node or spleen) from mature rats after neonatal thymectomy or sham thymectomy, or anti-thymocyte serum from birth

Treatment of animal providing lymphocytes for *stimulation* (from lymph nodes or spleen)	Mean DPM cultured separately	DPM in mixed culture	Net DPM in mixed culture	Net stimulation index (S.I.)	Mean net S.I.
A. *Complete Neonatal Thymectomy*					
Thymus-versus-lymph node	255	1025	141	0·5	
	* 111	83	102	0·9	1·1
	163	306	327	2·0	
Thymus-versus-spleen	150	271	336	2·2	
	* 104	83	118	1·1	1·8
	105	209	221	2·2	
B. *Incomplete neonatal thymectomy*					
Thymus-versus-lymph node	190	1397	1427	7·5	7·7
	* 36	287	289	8·0	
Thymus-versus-spleen	158	75	344	2·1	3·0
	* 37	137	148	4·0	
C. *Sham neonatal thymectomy*					
Thymus-versus-lymph node	142	714	677	4·8	6·9
	91	828	824	9·0	
Thymus-versus-spleen	* 37	124	134	3·6	3·2
	125	358	356	2·9	
D. *Anti-thymocyte serum*†					
Thymus-versus-lymph node	402	1334	774	1·9	1·9
Thymus-versus-spleen	559	1041	493	0·9	0·9

* Indicates results in which responses made 'one way' by X-irradiaton (1500 r) of *stimulating* lymphocytes from spleen or lymph node.

† 1 ml of anti-thymocyte serum given twice weekly to neonatal recipient for 4 weeks, and lymphocytes obtained 1 week later.

The present paper shows that there is also a gradient from thymus, to lymph node, to spleen, to bone marrow in the degree to which these various lymphocyte populations will stimulate allogeneic rat thymocytes, in a two-way or one-way MLR. However this finding may partly have resulted from contamination of the lymphocyte suspensions from spleen and bone marrow with fairly large numbers of red cells and other cell types. Nonetheless the anti-thymocyte serum experiments described, tend to confirm that the gradient relates to the proportion of thymus-derived lymphocytes. The strongest supportive evidence of

stimulation requirement for T cells (terminology of Roitt *et al.*, 1969) is provided by the neonatal-thymectomy studies. Similar results in thymus-deprived dogs have been reported by Kisken & Swenson (1969), but in their study, thymectomy was performed 3 years previously on adult animals of uncertain genetic disparity.

Major histocompatibility antigens appear to be represented upon all lymphocytes and not just T cells and hence the diminished stimulation capacity by B lymphocytes requires explanation. Differences in metabolic activity between B and T cells might account for the diminution shown. In rats Iversen (1969) has shown a higher basal rate of RNA synthesis in the non-recirculating lymphocyte population. Part of the proliferative response of normal human lymphocytes on mixed culture with lymphoma cell line lymphocytes in a 'one way' reaction has been attributed to the state of metabolic activation of the lymphoma cells (Hardy, Ling & Knight, 1969); and a positive response can still be obtained in autochthonous mixtures of normal lymphocytes and cell lines derived a year previously from the same donor (Steel & Hardy, 1970).

Preliminary cultures of thymocytes mixed in equal proportions showed maximal thymidine incorporation between the third and fourth days, but it could be argued that these conditions of culture were not optimal for stimulation by lymphocytes from nodes, spleen or bone marrow. However, neonatal thymectomy drastically reduced their stimulation capacity, and it seems unlikely that alteration in culture conditions would have materially influenced the results.

As an alternative hypothesis, it is suggested that a supplementary, membrane-related antigen system expressed only on thymus passaged lymphocytes could modify the existing histocompatibility antigen display on the T cell surface, by a subtle, spatial, reorientation, significant for cellular recognition but not generating any additional antibody specificities. As a consequence, B cells might present an intact but relatively inactive set of histocompatibility antigens, resulting in diminished stimulation capacity in the MLR.

Bone-marrow-derived lymphocytes of mice and rats also carry an extra surface antigen not shared by T cells (Raff 1970; Iversen 1970). This antigen might additionally have a modifying influence upon the histocompatibility antigen display of B lymphocytes. Expression of the T cell antigen on thymus passaging lymphocytes might involve either deletion or concealment of the B antigen.

The further concept that recirculating lymphocytes may carry a more active histocompatibility antigen display than that possessed by other tissues is supported by the experiments of Guttman, Lindquist & Ochner (1969). They showed that most of the immunogenic properties of rat kidney, transplanted between histoincompatible strains, resided in the contained lymphoid cells rather than the renal tissue itself. A similar conclusion can be drawn from the rat experiments described by Elkins (1964), who reported that local graft-versus-host reactions in the kidney were absent if recipient animals were first depleted of lymphocytes by preliminary lethal irradiation.

Lack of stimulant function by B lymphocytes in the MLR may partly explain the absence of maternal response to foetal lymphocytes and avoidance of host-versus-graft reaction against the foetus. Tissues of non-lymphoid origin appear to have only limited capacity to stimulate allogeneic lymphocytes (Hardy & Ling, 1969).

The increased thymidine incorporation found in autologous mixtures, appears to be a response of B lymphocytes to contact with thymocytes and is eliminated by prior X-irradiation of the non-thymic cells. It may indicate a relative paucity of T compared to

B lymphocytes in the rat bone-marrow environment, and supplementation may enable B lymphocyte response to circulating antigen in the serum used for medium supplementation.

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