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Interaction of Immune Lymphocytes with the Mixtures of Target Cells Possessing Selected Specificities of the *H*-2 Immunizing Allele

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Summary. Optimum conditions have been found for a specific quantitative absorption of mouse lymphocytes on to allogeneic target cells. Using this technique, it has been established that C3H anti-A lymphocytes immune to the specificities of a single *H*-2 sub-locus (D) were absorbed neither on cells bearing only some of the components of the immunizing complex (DBA/1 and I/St targets) nor on their mixture. Conversely, C57BL anti-A lymphocytes immune to the specificities of two *H*-2 sub-loci (D and K) reacted separately with each of the components on corresponding third-party targets (B10.D2(*H*-2d) and C3H (*H*-2k) as shown both by the direct cytotoxic effect and by the quantitative absorption technique. The results of absorption of these lymphocytes on mixtures of B10.D2 and C3H target cells used in various proportions indicate that C57BL anti-A lymphocytes represent a mixture of two 'polyvalent' populations in ratio of 1:3. This, together with the previous data indicates that 'committed' lymphocytes may be 'polyvalent' and that the initial recognition step of *H*-2 antigens may result from a direct contact between membranes of grafted cells and 'unprimed' host lymphocytes. Structural matching of the membrane antigenic complex and of normal and immune lymphocytes is suggested as a decisive factor of immunological recognition initiation in a transplantation context.

INTRODUCTION

Antigen-reactive cells (ARC) which do not release antibodies but are able to interact specifically with an antigen have been described recently (Mitchell and Miller, 1968; Taylor, 1968; Abdou and Richter, 1969). A strict clonality of these cells has been suggested on the basis of their ability to bind and to be made specifically tolerant by the corresponding antigen. Specific interaction of ARC with an antigen has been supposed to induce transformation of another cell line, precursors of antibody forming cells (P-AFC) into antigen-recognizing 'committed' cells (Y—or memory cells). The degree of clonality of a cellular type responsible for delayed-type hypersensitivity reactions and transplantation immunity is not known.

The cytotoxic effect of immune lymphocytes on allogeneic target cells may be used as an *in vitro* model for cellular immunity. This effect is seen through its two sequential steps: absorption of lymphocytes on the target during the first 6 hours of contact (Wilson, 1965) and the destruction of the target, requiring usually 20–40 hours (Rosenau and Moon,

1961; Brondz, 1964; Wilson, 1965). Specificity of the destructive action of immune lymphocytes may be seen by their quantitative determination on target cells of different genotypes (Brondz, 1968; Brondz and Golberg, 1970). Some peculiarities of this specificity have already been studied using lymphocytes immune to a single *H*-2 specificity or to several specificities. In addition, mixtures of such lymphocyte populations have been used. It was shown that the cytotoxic effect critically depends on the proportion of 'acting' lymphocyte receptors, rather than on the number of target specificities involved in the reaction. The cytotoxic effect was suggested to be the result of reaction with a complex of lymphocyte receptors as a whole, rather than of a number of separate events each involving interaction of individual receptors with the appropriate target specificity.

It was concluded from the results obtained that a lymphocyte immune to an *H*-2 antigen complex possesses receptors against the whole set of immunizing specificity and can be absorbed onto target cells provided only if all its receptors react with the target. It might be expected that such lymphocytes should interact neither with targets possessing only a selection of specificities of the immunizing complex nor with mixtures of such targets. This suggestion was examined in the present experiments using lymphocytes immune to antigens of one or two sub-loci of the *H*-2 system.

MATERIALS AND METHODS

Animals

Mice of inbred strains A/Mv(H-2^a), C3H/Sn(H-2^k), C57BL/10ScSn(H-2^b), B10.D2(H-2^d), DBA/1(H-2^q), I/St(H-2^l) were used at the age of 8–12 weeks.

Tumours

Polymorphocellular sarcomata were induced with methylcholanthrene in strain A mice and used in their third to twentieth passage generations. Tumour cell suspensions were obtained by trypsinization on the magnetic stirrer using 0·25 per cent trypsin solution with subsequent washing of cells with Hanks's solution.

Target cells

Primary cultures of mouse peritoneal macrophages were used as target cells. Macrophages were harvested 3 days after intraperitoneal injection of an irritant (5 ml of saline containing 2 per cent peptone and 0·1 per cent glycogen). Cells were suspended at a concentration of 2×10^5/ml in '199' medium with 20 per cent inactivated bovine serum, 10 per cent lactalbumin hydrolysate solution (0·5 per cent) and antibiotics, and were cultured for 2 days in flat-faced tubes containing 1 ml to obtain discontinuous uniform monolayer growth. When mixtures of macrophages of different mouse strains were used cells were harvested simultaneously and mixed immediately before culturing.

Experiments on the cytotoxic effect of immune lymphocytes were carried out as described before (Brondz, 1964, 1968). Briefly, regional lymph node cells were obtained 8 days after a single immunization with allogeneic tumour cells in five subcutaneous sites and intraperitoneally ($35–45 \times 10^6$ cells per mouse). After repeated washing the lymphocytes were suspended in 199 medium without serum, and $8–20 \times 10^6$ viable cells were introduced into the washed target-cell cultures. After 20 hours incubation at 37° the culture medium was replaced by 199 medium with 2 per cent bovine serum; the next day the living macrophages were counted and the cytotoxic index was determined as described before

(Brondz, 1965; Brondz and Golberg, 1970). Normal and immune lymphocytes from mice of the same inbred strain were used in each experiment. Each sample of lymphocytes was tested in four replicate cultures. The significance of the differences in results obtained was evaluated by Student's *t*-test.

Absorption of immune lymphocytes was performed at 27° on 3×10^6 target cells of different genotypes cultured in Carrel flasks, 7–8 cm in diameter. 5×10^7 or 10^8 lymphocytes suspended in 3 ml of 199 medium with 10 per cent bovine serum were introduced into each flask. After incubation the non-adherent lymphocytes were removed, centrifuged, suspended in 199 medium, and counted.

The decrease in cytotoxic activity of immune lymphocytes after absorption was then determined and compared with a sample of unabsorbed cells. For this, unabsorbed or absorbed immune and non-immune lymphocytes were incubated with fresh target cultures of appropriate macrophages in flat-faced tubes as described above. All samples were tested in parallel in each experiment using the same quantity of viable lymphocytes. The decrease in the cytotoxic effect of the absorbed lymphocytes was expressed by the absorption index (AI). AI was evaluated as $AI = \frac{a-b}{a} \times 100$, in which a and b indicate the cytotoxic effect (in per cent) of unabsorbed or absorbed lymphocytes from immune animals respectively. The same value defines the absorbing capacity of the given target. Target cells of the donor strain* (positive control) and of host strain* (negative control) were used for absorption in each experiment. (Details of calculation of cytotoxic effect and AI in the typical experiment are presented in Table 2.)

The distribution of *H*-2 specificities among the mouse strains employed are given in Table 1 according to Snell, Hoecker, Amos and Stimpfling (1964). Non-*H*-2 incompatibility can be disregarded because it does not influence the cytotoxic effect of lymphocytes in the conditions employed (Brondz, 1964).

Characteristics of the systems used

Two systems, C3H anti-A and C57BL anti-A were used, the lymphocytes being directed against eight *H*-2 specificities in each case (Table 1). The difference between these systems is that the target specificities are components of a single *H*-2 sub-locus (D) in the first case and of two *H*-2 sub-loci (D and K) in the second case. The latter is due to the fact that the C3H strain possesses specificities of one sub-locus (K) while C57BL mice lack almost all of them. Potential activity (PA) of immune lymphocytes was calculated as described earlier (Brondz and Golberg, 1970). As can be seen from Table 1, C3H anti-A lymphocytes immunized against eight specificities could potentially interact with five of them on DBA/1 target cells, with two on I/St target cells and with six when a mixture of DBA/1 and I/St target cells was used. C57BL anti-A lymphocytes immunized against eight specificities could potentially interact with five of them on B10.D2 target cells, five on C3H target cells and all eight when a mixture of B10.D2 and C3H target cells was used. The same lymphocytes could react with DBA/1 and I/St targets at the expense of four (or five) out of eight, and one out of eight specificities respectively. To study the interaction efficiency in the above mentioned combinations, the cytotoxic effect and lymphocyte absorption onto macrophages were determined quantitatively. Target cells of different genotypes were used in each experiment.

*Donor and host are referred to the original immunization.

TABLE 1

DISTRIBUTION OF *H-2* TARGET ANTIGENS OF THE TWO SYSTEMS EMPLOYED AMONG THIRD-PARTY TARGET STRAINS AND THEIR MIXTURES

System		C3H anti-A		C57BL anti-A	
H-2 sub-loci foreign to the host		D		D and K	
Immunizing *H-2* specificities		4, 6, 10, 13, 14, 27, 28, 29		1, 3, 4, 8, 10, 11, 13, 25	
Specificities shared by third-party strains	DBA/1	6, 13, 27, 28, 29	(5/8)	3, 8, 11, 13, 25?	(4/8)
	I/St	6, 10	(2/8)	10	(1/8)
	DBA/1+I/St	6, 10, 13, 27, 28, 29	(6/8)	—	
	B10.D2	—		3, 4, 8, 10, 13	(5/8)
	C3H	—		1, 3, 8, 11, 25	(5/8)
	B10.D2+C3H	—		1, 3, 4, 8, 10, 11, 13, 25	(8/8)

In parentheses: potential activity of immune lymphocytes (PA). PA is the ratio of the number of target specificities potentially capable of reacting with lymphocytes to the total number of specificities against which the lymphocytes are sensitized.

RESULTS

To determine the optimum conditions for the absorption of C57BL anti-A lymphocytes on A target cells the lymphocyte dose (5×10^7 or 10^8), the number of absorptions (one or two times) and time (1·3 or 6 hours) were varied. Each condition was duplicated. 75–85 per cent of the introduced lymphocytes remained non-adherent after incubation with target cells irrespective of lymphocyte dose, incubation time and target genotype. As can be seen from Table 2 and Fig. 1, the cytotoxic effect was reduced by 13–22 per cent in 1 hour and by about 40 per cent in 3 hours after inoculation, i.e. AI was 13–22 per cent and 40 per cent respectively (see Materials and Methods). AI exceeded 90 per cent after 6 hours incubation if the lymphocyte dose was 5×10^7, 6 hours incubation of the double lymphocyte dose (10^8) reduced their cytotoxic effect by 45–60 per cent only and two absorptions of this dose for at least 3 hours each were required to reduce the cytotoxic effect by 95 per cent. This inhibition of C57BL anti-A lymphocyte cytotoxic effect was specific because the same treatment (two times for 3 hours) by C57BL target cells either left the cytotoxic effect unchanged or reduced it not more than by 2–6 per cent, i.e. AI did not exceed 2–6 per cent (see Fig. 2). In all the following experiments lymphocytes in a dose of 10^8 were absorbed twice for 3 hours.

The results of two experiments on absorption of C3H anti-A lymphocytes by C3H, A, DBA/1, I/St and by the mixture of DBA/1 and I/St target cells are presented in Table 3. The cytotoxic effect of absorbed lymphocytes was reduced by 93·7 per cent (i.e. AI = 93·7 per cent) only when absorption was performed by donor target cells (A strain). Decrease of the cytotoxic effect by 21·6 per cent and −1·7 per cent after absorption by DBA/1 (PA = 5/8) and I/St (PA = 2/8) target respectively is insignificant as compared with AI obtained after the non-specific absorption by C3H target (13·8 per cent). Neither did the absorbing capacity (15·2 per cent) of DBA/1 and I/St target mixture (PA = 6/8) differ significantly from that of both the C3H target and of each of the mixture components. Thus, the mixture of target cells possessing selected but incomplete components of the immunizing complex is unable to absorb the lymphocytes immune to antigens of a single H-2 allele. In addition, these lymphocytes do not exert any direct cytotoxic effect on DBA/1 and I/St target cells as well as on their mixture (unpublished data).

TABLE 2

ABSORPTION OF C57BL ANTI-A LYMPHOCYTES BY A MACROPHAGES

No. of absorptions	Absorption period (hours)	Lymphocyte dose during absorption ($\times 10^6$)	No. of living target cells ($\times 10^3$) after incubation with 10^7 lymphocytes*		Cytotoxic effect (per cent)†	*P*	Absorption index (per cent)‡	*P*§
			Normal	Immune				
0	—	—	108·0±1·8	29·1±0·7	73·1	<0·001	—	—
1	1	50	—	39·7±2·8	63·3	<0·001	13·4	<0·02
1	3	50	—	65·7±1·3	39·1	<0·001	46·5	<0·001
1	6	50	—	101·4±1·7	6·2	>0·1	91·6	<0·001
0	—	—	90·4±6·8	17·9±4·1	80·2	<0·001	—	—
1	1	100	—	33·7±2·5	62·7	<0·001	21·8	<0·05
1	3	100	—	41·7±1·4	53·9	<0·001	32·8	<0·002
1	6	100	—	53·0±3·5	41·4	<0·001	48·4	<0·001
2	3+1	100	—	63·0±5·6	30·3	<0·01	62·2	<0·001
2	2+2	100	—	71·0±2·4	21·5	<0·05	73·2	<0·001
2	3+3	100	—	86·5±4·2	4·3	>0·1	94·6	<0·001

* Average of four tubes ± SE.

† $\frac{a-b}{a} \times 100$, in which a and b indicate the number of living target cells after incubation with normal (a) and immune lymphocytes (b) (mean of four tubes).

‡ $\frac{a-b}{a} \times 100$, in which a and b indicate cytotoxic effect of untreated (a) and absorbed immune lymphocytes (b).

§ Significance of the difference between mean number of living target cells after incubation with untreated and absorbed immune lymphocytes.

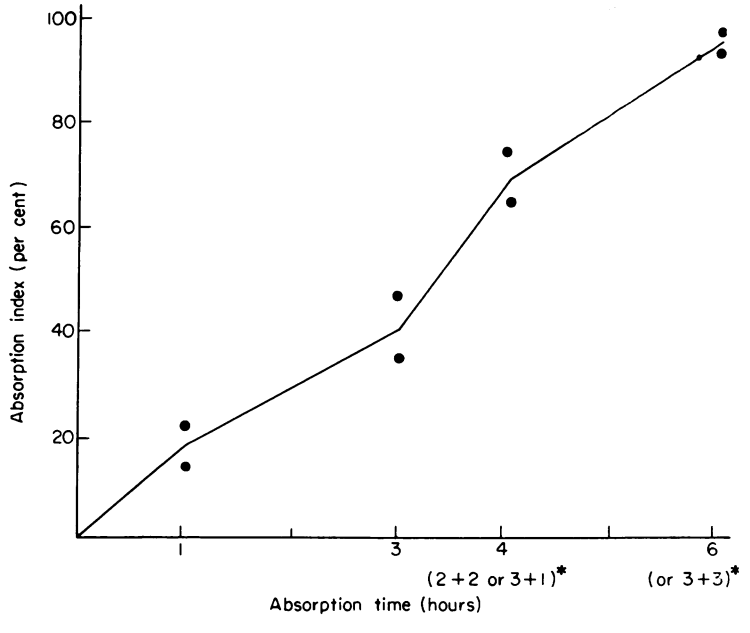

FIG. 1. Total data on the dynamics of immune lymphocyte absorption. Points indicate data of two experiments presented in Table 2. *Absorption was performed twice.

On the contrary, as can be seen from Table 4, C57BL anti-A lymphocytes were cytotoxic for not only A target cells (seven experiments) but also for B10.D2 and C3H third-party targets (six experiments) and for their mixture in a ratio of 1:1 (five experiments). The cytotoxic effect on B10.D2, C3H and B10.D2+C3H target mixture averaged 57, 92 and 92 per cent respectively as compared with 100 per cent on A target cells. When the lym-

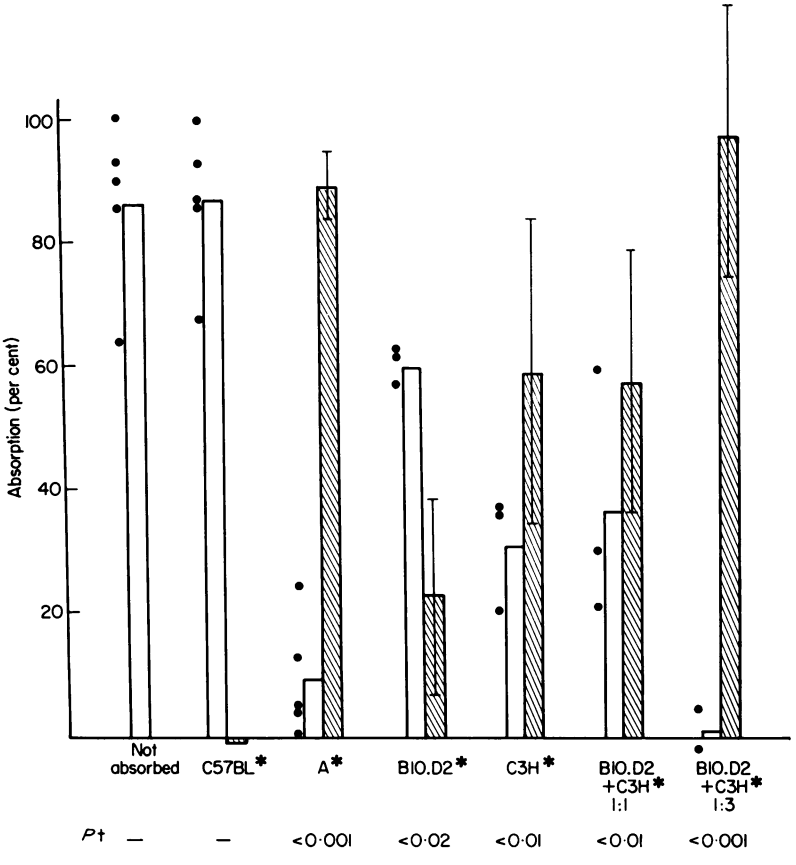

FIG. 2. Absorption of C57BL anti-A lymphocytes by macrophages of different genotypes* and by their mixture. Open columns, cytotoxic effect on A macrophages (per cent); hatched columns, absorption index.

† Significance of absorption, i.e. of the difference of the cytotoxic effects of lymphocytes absorbed by the given target and by C57BL unable to absorb specifically (separate data of each experiment). Points indicate data of the experiments, vertical bars are confidence limits at 95 per cent significance level.

phocyte dose was halved the cytotoxic effect upon either target reduced proportionally so that the correlations stated above changed only slightly. The same lymphocytes did not exert any significant effect on DBA/1 in two of three experiments (Table 4).

The result of absorption of C57BL anti-A lymphocytes by macrophages of different strains and by their mixtures (five experiments) are presented in Fig. 2. AI were 89·3 (75·6–100) per cent, 23(9·6–30) per cent and 59(42–77) per cent after treatment by A, B10.D2 and C3H macrophages respectively. In all cases AI were highly significant as

TABLE 3

ABSORPTION OF C3H ANTI-A LYMPHOCYTES BY MACROPHAGES OF DIFFERENT GENOTYPES AND BY THEIR MIXTURE

Origin of macrophages for absorption	Exp. No.	Cytotoxic effect on donor (A) target cells* (per cent)	Absorption index* (per cent)	P†
No absorption	1	73·2	—	—
	2	76·0	—	—
	Mean	74·6	—	—
A	1	9·3	87·3	$<0·01$
	2	0	100	$<0·01$
	Mean	4·7	93·7	—
C3H	1	45·9	37·3	—
	2	83·5	−9·8	—
	Mean	64·7	13·8	—
DBA/1	1	49·3	32·6	$>0·05$
	2	68·0	10·5	$>0·05$
	Mean	58·7	21·6	—
I/St	1	70·0	4·4	$>0·05$
	2	82·0	−7·9	$>0·05$
	Mean	76·0	−1·7	—
DBA/1+I/St	1	57·2	21·9	$>0·05$
	2	69·4	8·7	$>0·05$
	Mean	63·3	15·2	—

* See Table 2.

† Significance of the difference between cytotoxic effect of lymphocytes absorbed with the stated specific target and with C3H target which is unable to absorb specifically.

TABLE 4

CYTOTOXIC EFFECT (PER CENT) OF C57BL ANTI-A LYMPHOCYTES ON TARGET CELLS OF DIFFERENT GENOTYPES AND ON THEIR MIXTURE

Exp. No.	No. of lymphocytes ($\times 10^6$)	Origin of target cells				
		A	B10.D2	C3H	B10.D2+C3H 1:1	DBA/1
1	20	85·1	37·0 (43·5)*	60·5 (82·8)*	—	—
2	16	65·0	55.1 (84·8)	50·0 (77·0)	65·2 (100)*	23·4† (36·0)*
3	18	67·0	46·2 (70·0)	—	—	9·3‡ (13·9)
	9	41·4	17·5† (42·3)	—	—	—
4	18	93·0	68·5 (73·7)	100 (107)	85·4 (91·4)	10·0‡ (10·8)
5	18	73·7	25·0 (34·0)	72·8 (98·8)	71·4 (96·9)	—
6	20	80·2	47·1 (58·7)	66·6 (83·0)	67·4 (84·0)	—
	10	58·6	16·0† (27·3)	40·7 (69·5)	48·6 (83·0)	—
7	16	63·3	20·4 (32·2)	62·3 (98·4)	54·2 (85·6)	—
	8	37·2	—	36·6 (98·3)	33·0 (87·7)	—
Mean	16–20	75·3	42·8 (56·7)	70·4 (91·2)	68·6 (91·6)	14·2 (20·2)
	8–10	45·7	16·8 (34·8)	38·6 (83·9)	40·8 (85·2)	—

* In parentheses: per cent of cytotoxic effect with respect to that on A macrophages which is taken for 100 per cent.

† $P<0·05$.

‡ $P>0·05$; in other cases $P<0·01$.

compared with AI after the non-specific absorption by C57BL macrophages (average -0·3 per cent).

It can be seen from the AI data that the absorbing capacities of A, B10.D2 and C3H target cells with respect to C57BL anti-A lymphocytes are in the ratio of approximately 4:1:3. This can be interpreted to mean that 25 per cent of lymphocytes reactive to A cells are absorbed by B10.D2 target cells and 75 per cent by C3H target cells. If this were the case and if specificities of these two lymphocyte populations do not overlap lymphocytes reactive to B10.D2 and C3H targets should represent independent com-

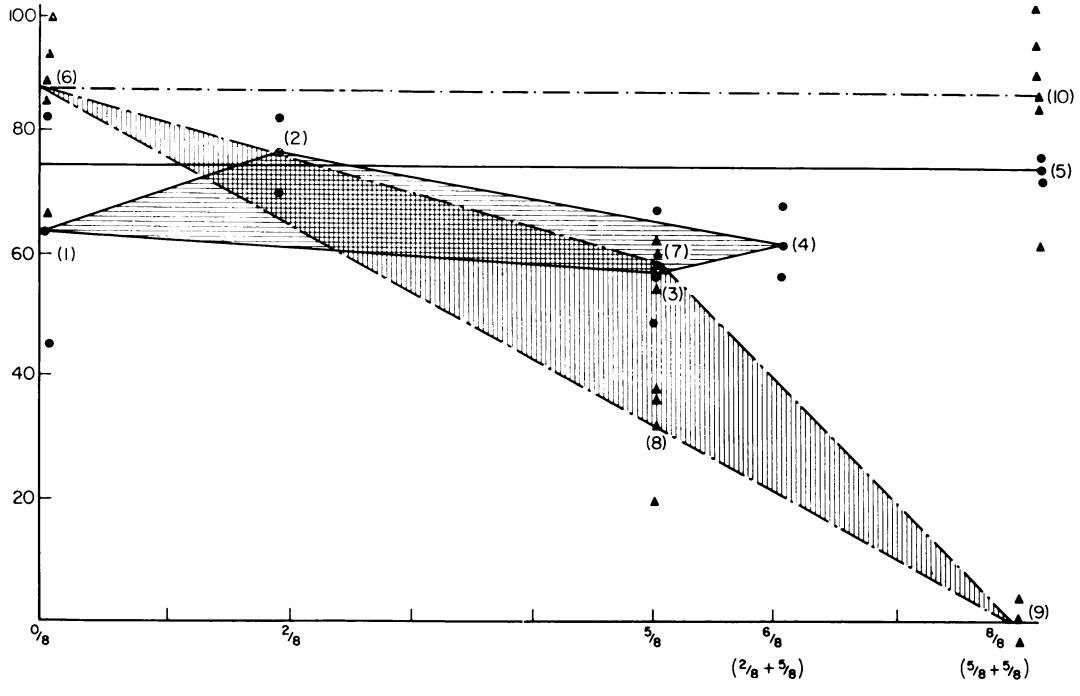

FIG. 3. Absorption of lymphocytes immune to antigen sets of a single allele (C3H anti-A) and of two alleles (C57BL anti-A) by macrophages of different genotypes and by their mixtures. x axis, proportion of lymphocyte receptors reacting potentially with the absorbing target; y axis, cytotoxic effect (per cent) of C3H anti-A lymphocytes (——) and C57BL anti-A ones (—·—) on A macrophages. C3H anti-A lymphocytes are absorbed by macrophages of C3H (1), I/St (2), DBA/1 (3), mixture I/St+DBA/1 (4), neither (5). C57BL anti-A lymphocytes are absorbed by macrophages of C57BL (6), B10.D2 (7), C3H (8), mixture B10.D2+C3H (9), neither (10).

ponents of C57BL anti-A suspension in the ratio 1:3. Therefore, the absorbing capacity of a mixture of B10.D2+C3H targets taken in the same ratio (1:3) should be about 100 per cent, equal to that of A target cells. However, the absorbing capacity of a 1:1 mixture of these target cells should not exceed the absorbing capacity of C3H target cells alone because the highly absorbing C3H target would by 'diluted' too much with the poorly absorbing B10.D2 target. The result obtained is in agreement with the expected one: absorption by 1:1 and 1:3 mixtures reduced the cytotoxic effect by 57·7(40–67) per cent and 97·5(95–100) per cent respectively (Fig. 2).

Total data on absorption of lymphocytes of different specificies are illustrated in Fig. 3. It can be seen that absorption by third-party target cells bearing different antigenic

components of the immunizing complex as well as by their mixtures does not essentially affect the cytotoxic activity of C3H anti-A lymphocytes immune to a single sub-locus complex but does inhibit the activity of C57BL anti-A lymphocytes immune to a two sub-locus complex.

DISCUSSION

It has been demonstrated in our previous reports (Brondz, 1968; Brondz and Golberg, 1970) that lymphocytes immune to an *H*-2 antigen complex were not absorbed by target cells possessing only a selection of the immunizing complex specificities (one out of four or two out of six). The same is shown to be the case in the present work when target cells possess two or five out of eight immunizing specificities. Poor specific absorption of immune lymphocytes in the case of target cells having an incomplete spectrum of specificities may be the consequence of at least two possibilities.

If lymphocytes immune to an *H*-2 antigen complex represented a mixture of cells each of which could react with only a single *H*-2 specificity only a small number of these cells should be absorbed by targets possessing an appropriately low representation of the immunizing specificities. Such a low extent of absorption may not be revealed because of the low sensitivity of the test. In this case, however, the absorption efficiency should be increased when using a mixture of targets possessing different components of the immunizing complex. This, however, has not proved to be the case: the absorbing capacity of the target mixture (DBA/1+I/St) with respect to C3H anti-A lymphocytes is as low as that of each of the components of the mixture and was considerably lower than that of donor (A strain) target.

On the other hand, if each lymphocyte had a complex of receptors reacting as a whole with the corresponding set of target specificities, such lymphocytes should be absorbed firmly neither by any component of the immunizing complex nor by a mixture of components. Our data are in agreement with this expectation and, thus, can be interpreted to mean that the bulk of lymphocytes immune to an *H*-2 antigen complex represent a 'polyvalent' population.

In the system described, all specificities of the immunizing complex represent components of a single *H*-2 sub-locus. Another feature of lymphocyte specificity appears to occur if the immunizing complex includes the specificities which are components of different *H*-2 sub-loci. It can be seen from the present findings that C57BL anti-A lymphocytes immune to antigens of two *H*-2 sub-loci (D and K), react with, in addition to A target cells, also with B10.D2 (H-2^d) and C3H(H-2^k) cells though these latter possess only a part of the specificities (five of eight) of the whole immunizing complex. One can further suggest from the quantitative absorption data that C57BL anti-A lymphocytes represent a mixture of two different populations, anti-D and anti-K, in ratio of 1:3. This suggestion was confirmed by the total absorption of these lymphocytes when B10.D2 and C3H target cells were mixed in the ratio of 1:3, but not of 1:1. On the other hand, DBA/1 target cells possessing only a part of the specificities of either D or K sub-loci were not significantly destroyed by C57BL anti-A lymphocytes. This indicates that an antigen mixture of two *H*-2 sub-loci induces polyvalent lymphocytes as well; in this case, however, the two populations appear to be immune to specificities of different alleles.

Our previous and present data taken together support the conclusion that lymphocytes are potentially able to become multispecific because (a) lymphocytes immune to an *H*-2

antigenic complex do not destroy the target cells possessing only a part of the corresponding specificities; (b) the mixture of lymphocytes immune to different but shared specificities of a third-party target are also inactive; (c) lymphocytes recognize a single target antigen provided that they have been immunized with this antigen only; (d) lymphocytes are absorbed neither by the third-party target cells possessing different selections of the immunizing complex specificities nor by mixtures of such targets; (e) lymphocytes immune to specificities of two *H*-2 sub-loci represent two populations directed against the antigens of the corresponding alleles.

Indications of polyvalent specificity of immune lymphocytes are in agreement with the capacity of lymphocytes (unlike plasma cells) to synthesize two or three classes of *H*-chain in the same cell as shown by blast transformation with the relevant antisera (Sell, 1967) and by immunofluorescence tests with cloned cultures of lymphoid cells (Takahashi, Takagi, Yagi, Moore and Pressman, 1969).

Specificities of lymphocytes immune to several antigens show mutual overlapping as demonstrated by the blast reaction induced by these antigens (Cowling and Quaglino, 1965; Caron, 1967). Multipotentiality of lymphocytes is a plausible explanation of an inordinately large proportion of primary reacting cells in the mixed lymphocyte cultures (Wilson, Blyth and Nowell, 1968).

The results obtained *in vitro* seem not to be in agreement with *in vivo* data which show that mice of one strain immunized against spleen cells of mice of a second strain display heightened resistance to skin grafts of a third strain (Berrian and Jacobs, 1959). However, the second-set rejection of a third-party graft can be explained by involving of non-*H*-2 specificities as a result of using non-coisogenic or such 'coisogenic' strains, the genetic disparity of which 'was found to include no less than four genes in either direction.' This is confirmed by the hastened rejection of third-party grafts even in some cases when *H*-2 specificities were not involved. Furthermore, the validity of Berrian's work is lessened by other shortcomings, namely, inhomogeneity in A strain, the opposite data in different mice within a particular group, the possible collaboration of humoral antibodies in the premature rejection of a third-party graft, etc. Our preliminary results (unpublished data) show that a third-party skin is not rejected prematurely in case of overlapping only for specificity 5 provided that the adequate conditions of the experiment are strictly followed.

According to the 'peripheral sensitization' hypothesis (Medawar, 1958) the recognition of transplantation antigens is initiated by circulating lymphocytes coming in contact with the foreign graft antigens rather than by the regional lymph node system. This suggestion has been confirmed when sensitization was performed with renal allograft (Strober and Gowans, 1965) or a hapten inducing the delayed hypersensitivity reaction (Macher and Chase, 1969). One can suppose that if antigens of a given *H*-2 sub-locus are localized together in limited regions of the cell membrane rather than scattered disorderly throughout the membrane, they may be recognized by peripheral lymphocytes as a single indivisible complex, thus giving rise to a single population of 'polyvalent' lymphocytes. On the other hand, antigens of two *H*-2 sub-loci may be localized in separate regions of the cell membrane. Such localization may provide recognition of each of the two antigens by different populations of normal lymphocytes, giving rise to two populations of immune lymphocytes as can be seen from the present work.

This suggestion is in agreement with the data on spatial separation of D and K antigens in the membrane of A strain cells (Boyse, Old and Stockert, 1968). Furthermore it has been shown recently that antigenic specificities representing members of a single *H*-2 allele

are not separable during elution from ion-exchange columns forming 'blocks' or 'inclusion groups' in which presence of any specificity appeared to be dependent upon the presence of others (Davies, 1969).

One can speculate that structural matching of the membranes of grafted cells and of unprimed circulating lymphocytes at their contact is a decisive factor responsible for recognition of *H*-antigens. This suggestion implies that pre-existing recognition sites specifically directed to different *H*-specificities may be located in the membrane of the same unprimed lymphocyte. This complex of sites, although perhaps variable before interaction with antigens, may become fixed on the lymphocyte membrane after contact with the corresponding antigen set (as suggested by Wilson *et al.*, 1968) and should be preserved during transformation of lymphocytes to blastic cells and the following differentiation to 'committed' small lymphocytes (Gowans, McGregor, Cowen and Ford, 1962; Oort and Turk, 1965). Conversely, maturation into antibody-forming plasma cells (Birbeck and Hall, 1967; Zlotnick, 1967) should be accompanied by the appearance of narrow specialization to the given antigenic determinant. This supposition is not in agreement with the hypothesis of clonal specificity of lymphocytes as far as transplantation immunity is concerned.

An alternative supposition would be that immune lymphocytes are directed against some intact configurational structure of target cell surface formed by a particular combination of *H*-2 specificities rather than against these specificities themselves. It was shown recently (Henney, 1969) that 'early' guinea-pig antibodies against human γ-globulin (unlike the 'late' ones) reacted only with a whole γ-globulin molecule but not with its fragments. Such possibility seems to be unlikely in cell-mediated immunity because (a) both a whole molecule and fragments of γ-globulin elicited the delayed hypersensitivity in the work mentioned, and (b) if configurational antigenic specificities at *H*-2 system really existed they should be detected by humoral antibodies. These, however, have so far not been shown.

The need for the whole complex of lymphocyte receptors to be involved in the reaction for effective absorption on target cells and for triggering an immune response might be explained on the basis of energy of binding: a contact of a fraction of receptors with appropriate target antigens would not be strong enough to provide needed binding energy. Such explanation seems to be unlikely since in delayed hypersensitivity to dinitrophenyl-polylysine almost all (96 per cent) the binding energy was provided by the hexamer complex (Schlossman and Levine 1967) which, however, appeared to be unable to elicit the cellular reactions.

The question whether recognition sites born by an immune lymphocyte have the same complex specificity or different narrow specificities is not one that can be answered at the moment. The capacity of lymphocytes to react only with the immunogenic heptamer complex of dinitrophenyl-polylysine in *in vivo* (Schlossman and Levine, 1967) and *in vitro* (David, 1969) delayed hypersensitivity reactions and the strict stereoisomeric specificity of these reactions (Schlossman and Yaron, 1970) favour the former possibility. Any further speculations on this subject would be premature.

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