Reports

Cell Mediated Immunity: Separation of Cells Involved in Recognitive and Destructive Phases

Abstract. *The mixed leukocyte culture (MLC) and the cell mediated lympholysis (CML) assays are used as in vitro models of the afferent, or recognitive, and efferent, or destructive, phases of the homograft reaction. Activity in both of these tests has been related to differences at the major histocompatibility complex, HL-A in man and H-2 in mouse. Recent evidence suggests that the presumed cell surface differences which lead to cell proliferation in MLC are different from those which act as a target for CML. Data are presented providing further support for this hypothesis; in addition separate cell populations may respond to the differences which activate cells in MLC and to the differences which serve as targets for CML. There thus appears to be a dichotomy both for genetic control of, and cell populations involved in, the recognitive and destructive phases of cell mediated immunity.*

Two in vitro models representing the afferent, or recognitive, and efferent, or destructive, phases of allograft reactions are the mixed leukocyte culture (MLC) (*1*) and cell mediated lympholysis (CML) (*2, 3*) assays, respectively. In MLC, recipient lymphocytes (for example, A) respond to foreign histocompatibility antigens on mitomycin C–treated donor cells (for example, B_m) by enlargement and division; response is measured by the incorporation of triated thymidine ([^{3}H]T) into the A responding cells. (We use capital letters to designate individual donors; for example, R, S, T, and X in Table 1, and G, H, I, and others in Table 2. We use A, B, and C to refer to three hypothetical donors for general discussion.)

If more thymidine is incorporated in an allogeneic mixture (AB_m) than in the control (AA_m), then donor and recipient differ for histocompatibility antigens. Lymphocytes from a mixed culture, AB_m, incubated for several days, acquire potential to damage ^{51}Cr-labeled target cells of B leading to ^{51}Cr release from these cells (the CML assay); this reaction is specific in that the same A lymphocytes usually cause less and in some cases no lysis of target cells from unrelated individuals: for example, C.

It has been generally assumed that MLC activation is due to differences between the two test cells for the serologically defined (SD) antigens controlled by the major histocompatibility complex (MHC), H-2 in mouse and HL-A in man. On the basis of unusual sibling pairs, Amos and Bach and their co-workers (*4*) suggested that human MHC components that are difficult to detect serologically may lead to MLC activation. This hypothesis has been strengthened by extensive studies in human families (*5*). In the mouse there are also MHC loci genetically separable from those determining the SD antigens, which, when different in two animals, will lead to MLC activation (*6*). Since to date it has been impossible to define the phenotypic products of these loci serologically, even though they lead to lymphocyte response in MLC, we refer to them as lymphocyte defined (LD) antigens. Thus, in both man and mouse, the MHC contains at least two types of loci which may be considered histocompatibility loci: one type controls SD antigens of HL-A and H-2, the other controls LD antigens.

The first evidence suggesting that LD differences of the MHC may not be sufficient to lead to the destructive phase of the allograft reaction came from studies in two strains of mice, B10.A(2R) and B10.A(4R). These two strains are genetically identical except for LD differences of the MHC (and other genes mapping with LD); their cells stimulate in MLC in one direction and show a splenomegaly graft versus host reaction in the direction predicted by MLC (*7*). There is, however, no skin graft rejection between these two strains. Additional evidence comes from studies by Eijsvoogel and his colleagues (*8*) who showed that, in one human family, LD differences alone led to MLC activation without subsequent CML. We have obtained similar results (*9*) in certain mouse strain combinations, including that of the B10.A(2R) and B10.A(4R)

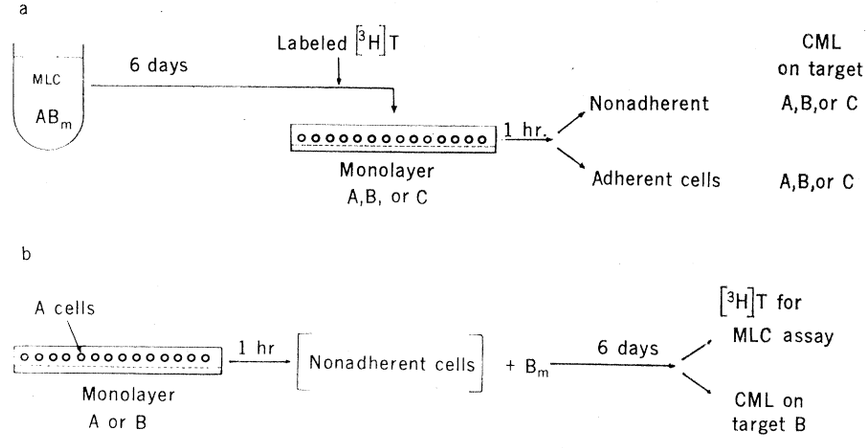

Fig. 1. (a) In the protocol, A cells are sensitized in MLC to B alloantigens. After 6 days of sensitization the cells are added to a monolayer of either A, B, or C adherent cells and left for 1 hour. The nonadherent cells are washed gently with the supernatant and removed from the monolayer; the adherent cells are collected as described in the text. The fate of cells incorporating tritiated thymidine in the MLC is studied by following the above protocol, except that the cells in MLC are labeled with [^{3}H]T for the last 5 hours in culture (before they are taken out of culture to be placed on the monolayer). In some experiments incorporation of [^{3}H]T into the nonadherent cells was studied after removal from the different monolayers. (b) In this protocol, "nonsensitized" cells A are adsorbed for 1 hour to a monolayer from A or from B; the nonadherent cells are then harvested and stimulated with mitomycin C–treated B cells. At the end of 6 days, different portions are assayed for incorporation of [^{3}H]T and for CML.

Table 1. Mixed leukocyte culture and cell mediated lympholysis studies in HL-A SD-identical unrelated individuals.

MLC	[^{3}H]T incorporated (count/min)	Percent CML ± S.D. in target cells*			
		Q	R	S	X
QQ_m	293 ± 97	−1.0 ± 4.8†			
QR_m	61,703 ± 5868	0.6 ± 4.6	4.1 ± 3.6	5.6 ± 1.0	5.4 ± 2.7
QS_m	48,213 ± 5202	8.1 ± 4.0	11.9 ± 2.7	13.4 ± 2.0	12.2 ± 3.1
RR_m	1,424 ± 1470		−2.5 ± 3.2		
RQ_m	75,983 ± 10616	12.5 ± 4.0	3.8 ± 3.5	6.5 ± 1.4	5.4 ± 1.4
RS_m	58,121 ± 7099	9.6 ± 4.5	−0.4 ± 3.6	2.3 ± 1.4	0.0 ± 1.9
SS_m	1,027 ± 119			0.6 ± 1.8	
SQ_m	91,792 ± 4369	26.6 ± 5.4	5.0 ± 3.1	7.0 ± 1.9	12.6 ± 2.0
SR_m	72,305 ± 3490	13.5 ± 3.8	6.7 ± 3.0	6.4 ± 2.2	7.0 ± 2.7
QX_m	92,575 ± 883	4.7 ± 4.3			26.1 ± 2.4
RX_m	98,307 ± 6058		0.5 ± 3.5		35.4 ± 6.2
SX_m	117,408 ± 8881			4.2 ± 1.7	27.8 ± 2.7
XX_m	708 ± 472				0.5 ± 3.7
XQ_m	84,422 ± 7787	52.7 ± 6.3	34.3 ± 4.4	33.5 ± 3.0	8.3 ± 7.2
XR_m	82,677 ± 2506	30.6 ± 7.5	19.7 ± 3.7	18.7 ± 2.3	5.5 ± 2.4
XS_m	84,681 ± 7447	59.1 ± 3.1	42.6 ± 4.4	38.8 ± 2.0	12.2 ± 4.1

* Cells Q, R, and S are HL-A 1, 2, 8, and 12. Cell X is HL-A 10 and 11. The ratio of effector cell to target cell in CML is 70 : 1. † The percentage of CML is calculated by the following formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

mentioned above. In both man and mouse the differences needed for CML are either the SD antigens themselves or a presumed cell surface component determined by genes closely linked to those determining the SD antigens.

We now present evidence that the dichotomy between MLC and CML holds not only in a family study in man and in inbred mouse strains, but also in unrelated human subjects, and further that the phenomenon may be explained by the presence of two separate cell populations—a hypothesis we have previously advanced (*10*). One population presumably proliferates in MLC in response to LD differences; the other mediates CML.

The MLC assay was performed as described (*11*). The cultures contained 0.1×10^6 to 0.3×10^6 responding and 0.2×10^6 to 0.3×10^6 mitomycin C–treated stimulating cells. The medium used was TC 199 (GIBCO) containing 20 percent human plasma. At the end of the culture period cells were either harvested for use as effectors in CML or labeled with [^{3}H]T. The CML assay was performed as described (*3*). Target cells were phytohemagglutinin–stimulated lymphocytes labeled with ^{51}Cr. Effector and labeled target cells were mixed at a ratio of 100 : 1 and incubated at 37°C for 4 hours. The mixtures were then centrifuged, the supernatants were collected, and the ^{51}Cr was released from the targets counted. The percentage of CML on a given allogeneic target cell must be compared with the control combination involving the same MLC tested on the target cell autologous with the responding cell in MLC. This control CML value can sometimes be significantly different from zero; such "autokilling" is not understood.

We have studied MLC and CML reactions in five unrelated individuals having the same four HL-A antigens, 1, 2, 8, and 12. Results of one experiment involving three of these individuals (Q, R, and S) are shown in Table 1. The MLC activation, assessed by [^{3}H]T incorporation, is strongly positive in all combinations, as shown in column 2, yet there is no CML in some combinations involving donors Q, R, and S and little or none in others. This contrasts with combinations involving donor X where cells Q, R, and S can either provoke (as stimulators in MLC) or provide (as effectors in CML) a good CML reaction.

The only case of positive CML among the HL-A–identical combinations is combination 8: that is, S cells sensitized to Q_m stimulating cells, tested on Q target cells. This could be explained either by heterogeneity of the antigens (that is, the cells do differ for SD) or by lympholysis directly against antigens other than SD. We have discussed this problem in the past in relation to a similar finding in two LD different mouse strains which are SD-identical, but give positive MLC and CML (*12*). We have already reported that cells of Q (donor K.M.) are significantly higher stimulators than other HL-A 1, 2, 8, and 12 cells with an HL-A 1, 2, 8, and 12 responder (*13*). The biological significance, if any, of this conjunction of exceptions is unknown.

These findings give further support to the concept that the LD differences that result in MLC stimulation do not necessarily serve as targets for CML. In addition, our results provide new and stronger evidence that the SD antigens, or the phenotypic product of a locus very closely linked to SD, are the targets for CML. If the products of alleles at such a hypothetical locus are the CML targets, the alleles must be in marked linkage disequilibrium with those of the SD loci.

Lymphocytes which mediate cytotoxicity adhere in vitro to target cell monolayers either prior to (*14*) or following sensitization (*15*). Experiments were designed to test whether cells activated in MLC can be separated into two populations by adsorption on monolayers.

Monolayers were prepared from cells that had been processed as described for MLC. Either 35×10^6 to 40×10^6 cells (in 5 ml) or 15×10^6 to 20×10^6 cells (in 2 ml) were plated on tissue culture plates (60 or 30 mm; Falcon), the medium being TC 199 as above. The cells were allowed to adhere to the dishes for 1 hour at 37°C. Nonadherent cells were then removed by gently pipetting the supernatant medium over the adherent cells, removing the medium and washing once with fresh medium. The MLC sensitized cells, 5×10^6 (in 3 ml) or 2×10^6 to 5×10^6 (in 2 ml), were incubated at 37°C for 1 hour on the fresh monolayer. Nonadherent cells were removed as described and used as effectors in CML. Cells adherent to the monolayer were collected by scraping with a rubber policeman; these preparations served as adherent cell effectors in CML.

Adherent cell monolayers were prepared from the individual whose cells were used as stimulating (sensitizing) cells in MLC, from the donor of the responding (effector) cells in MLC or from a third donor. Likewise CML was tested against target cells from the same three sources (Fig. 1a).

Results of three experiments are given in Table 2. No CML occurs unless the responding cells in MLC are stimulated with allogeneic cells. Nonadherent cells from the MLC GH_m adsorbed on a G monolayer and tested on H target cells give 46 percent CML. After adsorption on the H monolayer, the nonadherent cells give essentially no CML. (GH_m adsorbed on H monolayer and tested on H target cells gives

8 percent CML; however, GH_m adsorbed on H monolayer and tested on G target cells gives 7 percent CML.) Adsorption of the MLC GH_m on I monolayer and tested on H target cells gives 49 percent CML. In this experiment, GH_m adsorbed on G and tested on I did not give significant CML. In contrast to the removal of CML potential by specific adsorption, the nonadherent cells after adsorption on H monolayers do not contain significantly less [^{3}H]T (count/min).

In some cases adsorption on the specific monolayer did not totally remove CML potential from the MLC sensitized cells (Table 2). In this case specific adsorption of MLC JK_m on K monolayer cells reduced CML from 76 to 25 percent. Nonadherent cells from the MLC JK_m adsorbed on J monolayer did mediate CML against the L cells; this CML activity was significantly lowered by adsorption on either the K or L monolayers. Again, in contrast to removal of the CML reactive population on the K monolayer, [^{3}H]T present in the nonadherent population was not significantly reduced.

We have also tested the concept of separate cell populations following the protocol given in Fig. 1b. Results of one such experiment involving N cells adsorbed on either N or P monolayers are given in Table 2. After initial adsorption on P monolayer, N cells stimulated with P_m incorporate somewhat more [^{3}H]T (although not significantly so) in MLC than N cells adsorbed on N monolayer and then stimulated with P_m. Cytotoxic potential, however, is largely if not totally removed. (N cells adsorbed on a P monolayer, then stimulated with P_m, give 11 percent CML on P target cells; N cells adsorbed on P monolayer, then stimulated with N_m, gave 11 percent CML on P target cells.)

To summarize the results, we will refer all experiments to the general model of an MLC AB_m adsorbed on either A, B, or C monolayers and subsequently tested against B or C target cells. We have observed results similar to those in Table 2 in 11 experiments. In none of the experiments following the protocol in Fig. 1a were the cells in the nonadherent population which had incorporated [^{3}H]T significantly reduced by specific adsorption. In fact, in only three experiments was [^{3}H]T in the nonadherent cells lower after adsorption on the B monolayer than after adsorption on the A monolayer and in those cases not significantly so. Similarly, in neither of the two experiments following the protocol in Fig. 1b was the [^{3}H]T (count/min) reduced by adsorption on B monolayer as compared with A monolayer. To express this numerically, if the number of counts per minute of [^{3}H]T present in the nonadherent cells after adsorption of MLC activated cells on a B monolayer are divided by those present after adsorption on an A monolayer (for each experiment) and the result is expressed as a ratio, then the average ratio for all experiments equals 1.07, again indicating that few if any cells which divide during the period of labeling with [^{3}H]T are specifically adsorbed to the B monolayer. Similar results were obtained if [^{3}H]T was added to the nonadherent cells after adsorption, following the protocol for Fig. 1a.

In contrast to the lack of adsorption of these proliferating cells, a highly significant percentage, and in some cases all, of the CML potential was removed by adsorption on the specific B monolayer in every experiment. This was demonstrated both by the reduction in the nonadherent cells, sometimes to background levels, of CML potential after adsorption on B monolayers, and by the recovery from the B monolayer of CML effector cells. Yet, of the total [^{3}H]T present in the adherent plus the nonadherent cells, an average of only 9.5 percent was found in cells sticking to the B monolayer. When AB_m cells adsorbed on A monolayer gave significant CML on C target cells, this CML potential was removed in every experiment by adsorption on either B or C monolayer as compared with adsorption on A monolayer.

Cells responding in MLC can thus be operationally divided into two populations after adsorption on an adherent cell monolayer. The great majority of cells incorporating radioactive thymidine in the MLC are included in the nonadherent population; a highly significant percentage and in some cases the entire CML capability is removed by adsorption on the B monolayer.

It is possible that there is a minority population of dividing cells in MLC which are the CML effector cells. This finding is consistent with the somewhat higher number of counts per minute found on the B monolayer in some experiments. Our earlier findings that treatment of MLC's with bromodeoxyuridine and light, which kills dividing cells, abrogates CML (*16*) can be explained either if the CML effector cells divide or if the dividing nonadherent cells are needed to collaborate with the effector cells.

Table 2. Mixed leukocyte culture and cell mediated lympholysis studies after adsorption to monolayers. The percentage of CML is based on the spontaneous release (SR) and maximum release (MR) values (mean of triplicates ± S.D.) for each target cell as indicated in the footnotes.

Adsorbing monolayer	Nonadherent cells [^{3}H]T incorporation (count/min)	Target cell	^{51}Cr released (count/min)	CML (% ± S.D.)
	*GH_m as sensitizing MLC**			
G	27,439 ± 866	G	658 ± 70	6.1 ± 6.7
H	25,298 ± 1213	G	668 ± 53	6.9 ± 5.6
I	34,003 ± 1207	G	703 ± 48	9.7 ± 5.2
G	27,439 ± 866	H	1110 ± 29	46.0 ± 2.5
H	25,298 ± 1213	H	577 ± 20	8.3 ± 2.5
I	34,003 ± 1207	H	1162 ± 35	49.4 ± 2.9
G	27,439 ± 866	I	370 ± 8	5.2 ± 3.0
H	25,298 ± 1213	I	334 ± 17	1.4 ± 3.5
I	34,003 ± 1207	I	339 ± 19	2.0 ± 3.5
	JK_m as sensitizing MLC†			
J	12,139 ± 608	J	645 ± 59	18.9 ± 8.1
K	11,343 ± 790	J	579 ± 64	10.1 ± 8.8
L	12,671 ± 1136	J	614 ± 17	14.8 ± 3.4
J	12,139 ± 608	K	1332 ± 91	75.7 ± 9.3
K	11,343 ± 790	K	735 ± 43	25.0 ± 4.2
L	12,671 ± 1136	K	1308 ± 20	73.3 ± 28.7
J	12,139 ± 608	L	597 ± 99	79.3 ± 28.7
K	11,343 ± 790	L	369 ± 26	16.9 ± 7.7
L	12,671 ± 1136	L	392 ± 58	23.3 ± 16.2
	P_m as sensitizing cell after adsorption of N as effector cell‡			
N	12,112 ± 1742	P	358 ± 13	29.1 ± 3.5
P	16,457 ± 4297	P	227 ± 30	11.3 ± 5.2

*G:SR = 583 ± 49, MR = 1826 ± 31; H:SR = 458 ± 32, MR = 1884 ± 26; I:SR = 320 ± 29, MR = 1288 ± 17. † J:SR = 501 ± 22, MR = 1263 ± 40; K:SR = 442 ± 18, MR = 1617 ± 79; L:SR = 307 ± 9, MR = 673 ± 44. ‡ P:SR = 145 ± 26, MR = 877 ± 42.

The evidence presented provides support for the existence of two separate cell populations involved in recognitive and destructive phase of cell mediated immunity as studied in these in vitro systems. There is evidence that MLC responsive cells are largely thymus-derived (T) lymphocytes (*17*); investigations of monolayer-adherent sensitized cells and cells mediating cytotoxicity in systems similar to CML (*18*) also implicate T cells. Several lines of investigation suggest that more than one of type T cell exists (*19*). The separable cell populations reported here may thus represent two T cell populations. Alternatively, the CML active cell may be a bone marrow derived (B) lymphocyte (*20*) or a macrophage. One could argue that a single cell lineage is involved and that prior activation of LD antigens is required for that same cell to recognize SD and mediate CML. However, this interpretation is not consistent with the demonstration that adsorption of unsensitized cells on specific monolayers separates the cells responding in MLC from those mediating CML. This result, with evidence that MLC activation is required for CML (*9*), implies that cell-cell cooperation is involved in development of cells mediating CML.

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Dystrophic Chicken Muscle: Altered Synaptic Acetylcholinesterase

Abstract. *Individual motor endplates in the skeletal muscles of chickens genetically homozygous for muscular dystrophy have been compared with those in normal chickens. Measurements were made there, by specific autoradiographic techniques, of the numbers of total cholinesterase-like molecules and of acetylcholinesterase molecules. The acetylcholinesterase is distinctly decreased at the endplates in dystrophic muscles. The various data available on these muscles are compatible with the concept that a neural factor which determines the synaptic acetylcholinesterase, along with a number of other characters in the muscle cell, is defective in this disorder.*

Muscular dystrophy, in most of the types of this disorder in man and experimental animals, is inherited as a single Mendelian character (*1*); the molecular mechanisms, however, remain unknown. A major possibility that is consistent with several types of observation (*2*, *3*) is that regulation of functional systems in the muscle by a neural factor is genetically impaired. The synaptic junction is likely to be one of these regulated systems (*4*), and defects at this site in dystrophic muscles have been reported (*3*, *5*, *6*).

We report here on measurement of the cholinesterases (ChE) at the neuromuscular junctions in the muscles of normal chickens and of those homozygous for muscular dystrophy, revealing a difference there between the two types. The complement of cholinesterases [among which we include acetylcholinesterase (AChE)] is of especial interest here in view of the significant findings of Wilson and colleagues (*2*), that there are in these latter chicken muscles when dystrophy develops (soon after

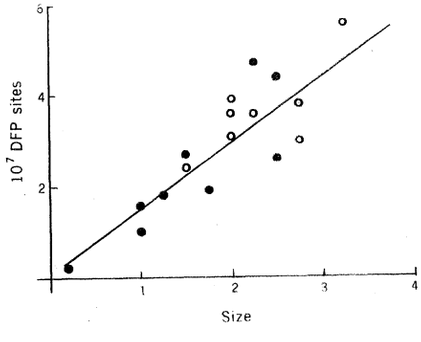

Fig. 1. The mean number of DFP-reactive active centers per endplate in the chicken muscles, measured by [^{32}P]DFP reaction and β-track autoradiography of endplates on individual fibers. These contents are shown as a function of the mean width of the fibers (in arbitrary units), both for normal (filled circles) and dystrophic (open circles) muscles: the increase in size of the fibers corresponds to the increasing age of the birds (to 14 months) or to dystrophy. Each point gives the mean value for the endplates of posterior latissimus dorsi or biceps muscle from one animal [a total of 254 fibers was employed, not including those for the lowest point (6-day chick) where 96 fibers in nondissected bundles were used]. A regression line was fitted by weighted least squares analysis, giving a correlation coefficient of $+.85$ ($P < .001$, for correlation).