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Tissue typing by mixed culture of lymphocytes. I. Demonstration of intracellular localization of transplantation antigen and H-2 antigen differences by the mixed cultures with addition of whole homogenate or ultracentrifuged fractions of mouse lymph-node cells

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Tissue typing by mixed culture of lymphocytes. I. Demonstration of intracellular localization of transplantation antigen and H-2 antigen differences by the mixed cultures with addition of whole homogenate or ultracentrifuged fractions of mouse lymph-node cells*

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Abstract

1. The cells used in the present experiments were lymph-node cells from inbred mice, and over 98 % cells were proven to be small lympho-cytes. Therefore, those cells that have undergone blastformation are all those derived from small lymphocytes. 2. When homogenate of one cell group is cultured with live cells of the other pairing group, there occurs blastformation. In the presence of PHA, such a blastformation becomes more marked. 3. The optimal concentration of PHA (phytohemagglutinin)-M added to the mixed culture is found to be 1% (v/v). 4. The maximum rate of blastformation in the mixed culture is observed at the culture hour 48, being much faster than in the mixed culture between two live cell groups. 5. In the mixed cultures between subcellular fractions prepared from cell homogenate by centrifugation and live cells, the transplantation antigenic potential (histocompatibility antigenic potential) is seen in the mitochondrial and the microsomal fractions, especially marked in the latter. 6. In the observations carried out by various combinations of these inbred mice, it has been demonstrated that the rate of blastformation induced by the addition of cell homogenate or sediment fractions prepared from the homogenate reflects quite accurately the differences in H-2 antigens.

TISSUE TYPING BY MIXED CULTURE OF LYMPHOCYTES
I. DEMONSTRATION OF INTRACELLULAR LOCALIZATION OF TRANSPLANTATION ANTIGEN AND H-2 ANTIGEN DIFFERENCES BY THE MIXED CULTURES WITH ADDITION OF WHOLE HOMOGENATE OR ULTRACENTRIFUGED FRACTIONS OF MOUSE LYMPH-NODE CELLS

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It is known that so long as the major histocompatibility antigens of donor and recipient are compatible, the transplant can survive for a long period of time and the difference in the minor histocompatibility antigens can be eliminated by the available routine immunosuppression. Therefore, in order to accomplish successful organ transplantation the minimum requirement is to eliminate the donor possessing the major histocompatibility antigen. In view of this, the mixed lymphocyte culture test (MLC-test) had so far been used as one of the methods for tissue typing, but the evidence was lacking as to whether or not this test was appropriate for tissue typing. In the MLC-tests conducted with regional lymph-node cells of inbred mice having known H-2 antigens (the major histocompatibility antigen) in the presence of phytohemagglutinin (PHA), KANEDA of our laboratory has demonstrated that the blastformation of lymph-node cells faithfully reflects the differences in H-2 antigens (1, 2, 3), proving that MLC-test is applicable to tissue typing.

However, since lymph-node cells of either donor or recipient are whole cells, and the test is not one way stimulation test, there is no denying that it is somewhat not suitable for selecting proper donor.

For the purpose to clarify this problem further series of mixed lymph-node cell cultures were conducted by one way stimulation method in which the groups of donor lymph-node cells were destroyed and cultured with the recipient groups of living lymph-node cells of mice. As a result it has been demonstrated that the lymph-node cells of the recipient group show blastformation with a significant difference, proving clearly the differences in H-2 antigens. This communication deals with the findings of such a study.

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METERIALS AND METHODS

Materials

1) *Animals:* The mice used were A (H-2ᵃ), C57BL (H-2ᵇ), CBA (H-2ᵏ) and AKR (H-2ᵏ), 2—3 months old, all obtained from The Mouse Colony of Okayama University Medical School.

2) *Culture medium:* Hanks solution, Earle solution supplemented with 3.0 g lactalbumin hydrolysate and 1.0 g yeast extract per 1000 ml (YLE), TC-199 and calf serum were obtained from Chiba Serum Research Laboratory.

3) *Antibiotics:* Each of penicillin-G potassium sulfate and Kanamycin sulfate in the dilution of 1,000 μ/ml and 1 mg/ml dissolved in Hanks solution was added to the medium in the ratio of 150 units/ml and 150 r/ml final concentration.

4) *PHA:* Using Bacto-phytohemagglutinin -M (Difco), by dissolving this 50 mg in 5 ml TC-199 solution, it was used as 100 % (v/v) PHA-M solution. Just before the use it was further diluted with TC-199 to 1 % (v/v).

5) *Centrifugal apparatus:* For ultracentrifuge, Hitachi RP-40 rotor was used.

Methods

1) *Preparation of lymph-node cell suspensions*

i) *The collection of lymph-node cells:* The neck and axillary lymph nodes of mouse are taken out, cut into small pieces in 10 ml of Hanks solution, left standing for 30 minutes to allow the lymph-node cells to wander out. Then this cell suspension is passed through the 80-mesh filter, the filtrate is centrifuged at 1,500 rpm for 10 minutes, to the sediment thus obtained 10 ml Hanks solution is added, it is stirred well with a glass pipette to have the cells resuspended in the solution, and this suspension is again subjected to the centrifugation as before. These procedures are repeated 3 times to remove thoroughly the liquid components, then to the sediment which is the cell component, 5 ml TC-199 solution is added, stirred well, and with a portion of this cell suspension the cell count is taken.

ii) *Determinaiton of cell survival:* To a portion of the cell suspension as prepared in the foregoing, 0.5 % Trypan blue is added, those cells stained deep blue are taken as dead cells and those unstained ones as the live cells, the cell counts are taken as soon as possible after the staining, and the live cell suspension serves as the material. By the above procedures 98 % (in average) of the cells are prove to be live ones.

iii) *Preparation of cell samples:* After counting the cell number with Bürker-Türk's hemocytometer, an appropriate amount of TC-199 solution is added to the cell suspension adjusting the cell concentration to 10^7 cells/ml to be used in the subsequent experiments.

iv) *Determination of the cell size:* With each lymph-node cell sample prepared a portion of it is picked up with a rod and by staining these lymph node cells with May-Grünwald-Giemsa stain the cell size is determined according to the classification mentioned later. As a result it has been found that more than 98 % of each sample proves to be small lymphocytes.

2) *Preparation of whole cell homogenate*

The glass and Tefron homogenizer that have been previously sterilized thoroughly for 48 hours with 0.01% Osborn's solution are washed well with Hanks solution kept at 4°C to remove the Osborn's solution.

After washing, 5 ml of the cell suspension adjusted to contain 10^7 cells/ml is put into the glass homogenizer, then the homogenizer is revolved at full speed making one round 2 minutes each and repeating this for 7 rounds to the total of 15 minutes to destroy the cells completely. Next, this is transferred to the Tefron homogenizer and the cells are further destroyed for 15 minutes just as in the case with glass homogenizer. The glass and Tefron homogenizer when destroying the lymph-node cells and subsequent cell homogenate are kept in a jar over the ice bath. The destroyed lymph-node cell substance serves as the 10^7 cells/ml homogenate for the subsequent experiments.

3) *Preparation of subcellular fractions*

To the lymph-node cell suspension 0.25 mole sucrose solution is added, this mixture is homogenized with glass homogenizer and with Tefron homogenizer both in the ice bath, then the homogenate so prepared is fractionated by the Schneider's method of liver cell centrifugal fractionation.

At first the cell homogenate is centrifuged at $700 \times g$ for 10 minutes, of which the supernatant (1) and the sediment are separated. To the sediment is added 10 ml of 0.25 mole sucrose solution, then centrifuged at 700—1,000 rpm for 5—10 minutes to remove live cells and tissue components, the supernatant is centrifuged at $700 \times g$ for 10 minutes of which the supernatant (2) and the sediment are separated. This sediment which contains mainly nuclear fraction is taken as F_1 fraction.

Next, the supernatants (1) and (2) are mixed, centrifuged at $8,500 \times g$ for 15 minutes to separate the mixture into the supernatant and the sediment. The sediment so obtained contains principally the mitochondrial fraction and is designated as F_2 fraction.

The supernatant separated here is transferred to the polyethylene super-centrifugal tube and is further centrifuged at $10 \times 10^4 \times g$ for 60 minutes to separate
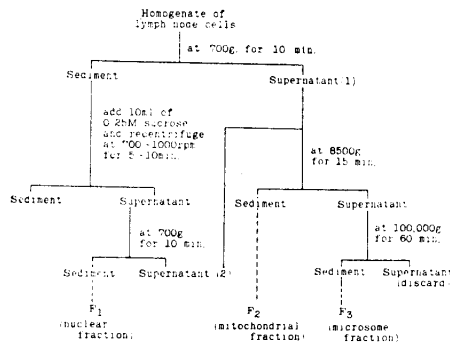

Fig. 1. Fractions of mouse lymph node cell homogenate (in isotonic sucrose solution)

the sediment. This sediment contains mainly microsomes and is taken as fraction F_3 (Fig. 1).

Each of these fractions, F_1, F_2, and F_3, is kept in ice cold condition and is considered to contain the subcellular fractions of the entire number of the original lymph-node cells. TC-199 solution is added to each of these sediments, then to each fraction solution, lymph-node cells are resuspended so as to contain 4×10^7 cells in 1 ml of each fraction solution.

4) *Cell culture and preparation of smears*

i) *Cell culture :* The medium is composed of Hanks solution, YLE, calf serum and TC-199 solution (1 : 2 : 2 : 5, v/v). In the case of mixed culture 10^6 lymph-node cells and homogenate are mixed in the volume 1 : 1, and in the case where the sediment fraction is used, 10^6 lymph-node cells/ml is mixed with the sediment in the ratio of 1 : 4 (v/v), then added into the medium. For the control groups, lymph-node cells only are cultured. These mixed or single cell cultures are consisted of the groups added with PHA and those without PHA. Further, every culture tube contains 150 units/ml of sulfate penicillin-G and $150\,r$/ml of sulfate Kanamycin. To do so, set 9 tubes per series and incubate them at 37°C, inclined at the angle of 15° in the incubator.

ii) *Preparation of smears*

After 24, 48 and 72 hours of the cell cultures as mentioned in the foregoing, 3 culture tubes each are taken out, the content of the tube is transferred to Spitz glass, diluted with Hanks solution, centrifuged at 800 rpm for 10 minutes, by decanting the supernatant in such a way as to leave a little of the supernatant, stirring the sediment well with a micropipette, droplets of the cell suspension are placed on the slide glass, several smears are drawn. After drying it is stained with May-Grünwald-Giemsa stain, washed with water, and dried to serve as the sample for microscopic observations.

5) *Determination of the size of cultured cells*

i) *Standard of identification :* The size of mouse lymph-node cells is identified according to the classification of TANAKA *et al.* (1, 2) and KUROYANAGI (4) (Table 1); namely, the percentage of large and intermediate lymph-node cells is taken as the rate of blastformation.

Table 1   Standard for the determination of the size of lymphocytes (mouse)

small lymphocyte	$<56\,\mu^2$
intermediate lymphocyte	$56\,\mu^2 < < 110\,\mu^2$
large lymphocyte	$>110\,\mu^2$

$$\text{rate of blastformation} = \frac{\text{the number of intermediate} + \text{large lymphocytes}}{\text{the total number of lymphocytes}} \times 100$$

ii) *Determination of cell size :* In the determination of the cell size on the slide glass we use the object lens of $100\times$ and the eye-piece of $10\times$, by oil immersion method. In this instance, a glass-calibrated into 50 equal parts is put inside the eye-piece, and the dimensions of the cells are taken both crosswise and vertically

to calculate the area and they are classified into large, intermediate and small cells according to the standard identification.

<div align="center">RESULTS</div>

a) *Study on the culture conditions*

1. *Changes in the number of cells in culture with lapse of time*

Observations were carried out with the group of single culture without PHA to see how the number of 10^6 cells/ml of lymph-node cells placed in each culture vessel would change with lapse of time. For this purpose, each culture vessel at the beginning of the culture, and 24 hours to 7 days later is taken out, shaken well with Vibro shaker, and 0.2 ml of the cell suspension is taken out and mixed with 0.8 ml crystal violet, incubated at 37°C for 30 minutes, stirred again with Vibro shaker, and a portion of this stained suspension is taken out with a micropipette and the number of the cells is counted by Bürker-Türk's hemocytometer.

As shown in Fig. 2, 10^6 cells/ml at the beginning of the culture
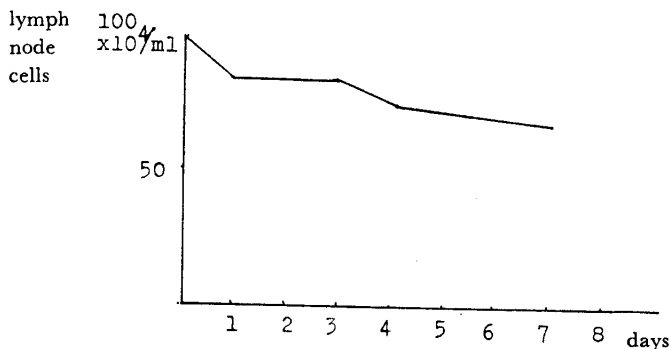

Fig. 2 Change in the cell number with lapse of time in single cell culture

decreases very rapidly to about 80×10^4 cells/ml after 24-hour culture, but thereafter the number is kept almost unchanged during the second and the third day, and 4 days later the number gradually decreases, and by the seventh day it has decreased to 70×10^4 cells/ml. During the whole course of this period no change such as exchange of the medium has been done.

2. *Changes in the rate of blastformation*

In the observations carried on regarding the changes in the rate of blastformation up to 96-hour culture with the single and the mixed cultures of the groups with 1 % (v/v) PHA and those without PHA, by the 24-hour culture time a considerable rate of blastformation could be observed

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in the combinations anticipated to show a strong reaction, but the periphery
of the cell bodies at that time being indistinct, it was not suitable for
calculating the area of the cells. By the 48-hour culture the blastformation
reached its maximum rate in the mixed cultures. In the subsequent 72-
hour and 96-hour cultures the periphery of the cells was fairly distinct,
but some cells showed broken cell boundary, as well as a decrease in the
rate of blastformation and poor stainability.

On the other hand, in the groups of the single cell culture, differing
from those of the mixed culture, the maximum rate of blastformation
was observed up to the culture hour 96. These results suggested that the
appropriate time to compare the rate of blastformation would be at the
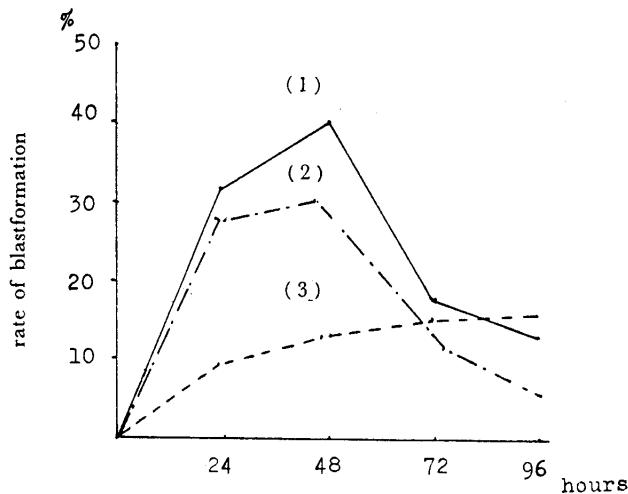culture hour 48 (Fig. 3).

Fig. 3   Changes in the rate of blastformation with lapse of time in mixed
cultures of sediment of homogenized cells and untreated allogeneic cells
  (1)   CBA live lymph node cells+A 100,000×g sediment with 1% (v/v) PHA-M
  (2)   CBA live lymph node cells+A 8,500×g sediment with 1% (v/v) PHA-M
  (3)   CBA live lymph node cells with 1% (v/v) PHA-M

3.   *Concentration of PHA*

Of the mixed cultures in the combination of CBA live lymph-node
cells+A lymph-node cell homogenate the rate of blastformation was com-
pared with the group cultured with PHA-M at the concentration of 0.5%
(v/v), 1% (v/v) and 3% (v/v). As a result it was found that the highest
rate of blastformation was observed in the addition of PHA-M at the
concentration of 1% (v/v). Therefore, all the subsequent addition was
invariably set at the concentration of PHA-M 1% (v/v) (Fig. 4).

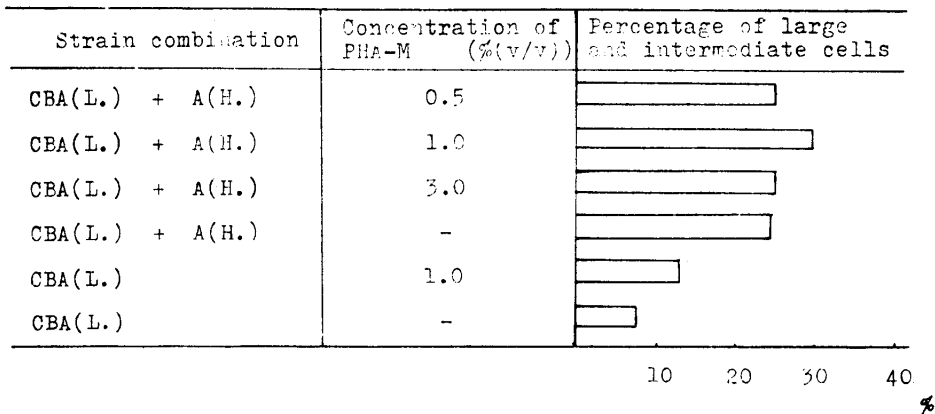| Strain combination	Concentration of PHA-M (%(v/v))	Percentage of large and intermediate cells
CBA(L.) + A(H.)	0.5	
CBA(L.) + A(H.)	1.0	
CBA(L.) + A(H.)	3.0	
CBA(L.) + A(H.)	-	
CBA(L.)	1.0	
CBA(L.)	-	

Fig. 4 Relationship between the concentration of PHA-M and the rate of blastformation
(L.) : live lymph node cells
(H.) : whole homogenate of lymph node cells

b) *Experiments proper*

1. *Single cell culture groups*

This single culture is always carried out along with mixed culture as to serve the control. Looking at the comparative rate of blastformation between the single culture group with 1 % (v/v) PHA and the group without PHA, the average rate in the groups with PHA is 13.5 % while it is 6.9 % in the groups without PHA, as illustrated in Table 2.

Table 2 Percentage of large and intermediate cells in single cell cultures

Experiment No.	Strain	Percentage without PHA-M	Percentage with 1% (v/v) PHA-M
1	A	5.0	10.2
2	A	7.0	17.3
3	A	8.2	11.8
4	C57BL	7.6	12.2
5	C57BL	8.8	16.7
6	C57BL	5.0	12.6
7	CBA	7.4	11·6
8	CBA	7.0	13.6
9	CBA	5.6	13.2
10	CBA	7.5	12.0
Average		6.9	13.5 ($p < 0.001$)

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2. *Mixed culture groups*

i) *With whole homogenate*

In the mixed cultures of live lymph-node cells and the whole homo-genate mixed in the ratio of 1 : 1, either with or without PHA, the results are as shown in Table 3.

Table 3   Percentage of large and intermediate cells in mixed cultures of
homogenized cells and untreated allogeneic cells

Strain combination	Percentage without PHA-M	Percentage with 1% (v/v) PHA-M
CBA(L.) + A(H.)	24.0 (p<0.001)	28.0 (p<0.001)
CBA(L.) + C57BL(H.)	26.4 (p<0.001)	31.0 (p<0.001)
A(L.) + AKR(H.)	17.5 (p<0.001)	20.3 (0.01<p<0.02)
CBA(L.) + AKR((H.)	12.8 (0.02<p<0.05)	16.2 (0.1<p<0.2)
AKR(L.)+ AKR(H.)	8.8 (0.8<p<0.9)	12.6 (p>0.9)

(L.) : live lymph node cells
(H.): homogenized lymph node cells

In such combinations as A or C57BL homogenate+CBA live lymph-node cells with 8 different H-2 antigens (Table 4), there can be observed

Table 4   The known alleles and isoantigenic specificities of the H-2
system and the inbred strains that carry them

H-2 alleles	H-2 antigenic phenotype (isoantigen)												Inbred strains
a	A	C D E F	H J K M N	Y A'B'C'								A
b	D^b	E F	N V	A'B'C'	K^b							C57BL
k	A	C	E	H	K	Y	D^k					CBA, AKR, C3H

(Snell, G. D. *et al.*, 1964)

about 30 % of blastformation, whereas in the combination of AKR homogenate+A live lymph-node cells with 6 different H-2 antigens, the blastformation can be seen in about 20 %, and also in the AKR homo-genate+CBA live lymph-node cells where there is no difference in H-2 antigen, the rate of blastformation is about the same or slightly higher than that of the controls. In the control proper of the AKR homogenate+ AKR live lymph-node cells the results are similar to those of the single cell culture.

ii) *With ultracentrifuged fractions*

The blastformation rate in the mixed culture of live lymph-node cells with ultracentrifuged fractions in the ratio of 1 : 4, also reaches its peak

at 48 hours later.   Therefore, the results of this combination are all taken at this time.

In the combination of A (sediment fraction)+CBA (live lymph-node cells) where the difference in H-2 antigens is major the results of each fraction are as shown in Fig. 5.   Looking at the groups cultured with

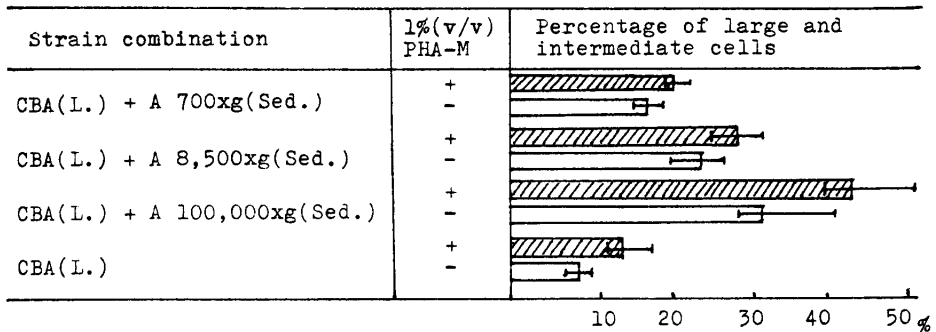| Strain combination	1%(v/v) PHA-M	Percentage of large and intermediate cells
CBA(L.) + A 700xg(Sed.)	+ −	
CBA(L.) + A 8,500xg(Sed.)	+ −	
CBA(L.) + A 100,000xg(Sed.)	+ −	
CBA(L.)	+ −	

10   20   30   40   50 %

Fig. 5. Antigenic activities of different sediments
(L.)   : live lymph node cells
(Sed.) : sediment of the homogenate of lymph node cells

PHA, the blastformation rate with $700 \times g$ sediment is 19.9 %, and with 8,500 $\times g$ sediment 28.0 %, with 100,000 $\times g$ sediment 43.6 % indicating the presence of strong antigenicity in the 100,000 $\times g$ sediment. In addition, in the groups cultured without PHA results are similar (Fig. 5).

Table 5 Percentage of large and intermediate cells in mixed cultures of $700 \times g$ sediment of homogenized cells and untreated allogeneic cells

Strain combination	Percentage without PHA-M	Percentaeg with 1% (v/v) PHA-M
CBA(L.) + A(Sed.)	16.8 ($p < 0.001$)	19.9 ($p < 0.001$)
CBA(L.) + C57BL(Sed.)	16.7 ($p < 0.001$)	18.8 ($p < 0.001$)
C57BL(L.) + A(Sed.)	16.4 ($p < 0.001$)	19.2 ($p < 0.001$)
A(L.) + AKR(Sed.)	9.9 ($p < 0.001$)	11.8 ($0.6 < p < 0.7$)
CBA(L.) + AKR(Sed.)	11.7 ($0.001 < p < 0.01$)	12.8 ($0.4 < p < 0.6$)

(L.) : live lymph node cells
(H.) : $700 \times g$ sediment of homogenized cells

Next, in attempts to see the rate of blastformation by fractionating alternately those cells with major difference and those with minor differ-ence in H-2 antigens, and culturing them with sediment factions and live lymph-node cells, the following results were obtained. With $700 \times g$ sedi-ment fractions, the combinations where H-2 antigen difference is major

show much greater blastformation than the control, but in the combinations where the H-2 antigen difference is minor, the results resemble those of the controls (Table 5).

Table 6 Percentage of large and intermediate cells in mixed cultures of 8500×g sediment of homogenized cells and untreated allogeneic cells

Strain comcination	Percentage without PHA-M	Percentage with 1% (v/v) PHA-M
CBA(L.) + A(Sed.)	22.8 (p<0.001)	28.0 (p<0.001)
CBA(L.) + C57BL(Sed.)	22.3 (p<0.001)	25.5 (p<0.001)
C57BL(L.) + A(Sed.)	20.2 (p<0.001)	25.9 (p<0.001)
A(L.) + AKR(Sed.)	12.6 (p<0.001)	14.8 (0.2<p<0.3)
CBA(L.) + AKR(Sed.)	13.7 (0.002<p<0.01)	15.1 (0.1<p<0.2)

(L.)   : live lymph node cells
(Sed.) : 8500×g sediment of homogenized cells

With 8,500×g sedimeet fractions, the combinations such as CBA (live lymph-node cells)+A (sediment fraction), CBA (live lymph-node cells)+C57BL (sediment fraction) and C57BL (live lymph-node cclls)+A (sediment fraction) with 8 different H-2 antigens, show the rate of blast-formation as high as about 30%, whereas in A (live lymph-node cells)+AKR (sediment fraction) with only one different antigen, and in CBA (live lymph-node cells)+AKR (sediment fraction) without H-2 antigen difference, the rates are only slightly higher than that of controls, indicating that such mixed cultures can sufficiently help us to identify differences in H-2 antigens among different inbred mice (Table 6). As for the 100,000×g sediment fractions, the increase in the rate of blastformation is far more marked, enabling us to identify the differences in H-2 antigens still more distinctly (Table 7).

All these values are the average of at least 3 trials or more each.

Table 7 Percentage of large and intermediate cells in mixed cultures of 100,000×g sediment of homogenized cells and untreated allogeneic cells

Strain combination	Percentage without PHA-M	Percentage with 1% (v/v) PHA-M
CBA(L.) + A(Sed.)	31.1 (p<0.001)	43.6 (p<0.001)
CBA(L.) + C57BL(Sed.)	27.5 (p<0.001)	33.3 (p<0.001)
C57BL(L.) + A(Sed.)	28.0 (p<0.001)	33.2 (p<0.001)
A(L.) + AKR(Sed.)	15.8 (p<0.001)	17.8 (0.02<p<0.05)
CBA(L.) + AKR(Sed.)	14.9 (0.001<p<0.01)	17.7 (0.02<p<0.05)

(L.)   : live lymph node cells
(Sed.) : 100,000×g sediment of homogenized cells

DISCUSSION

Studies on the blastformation of lymphocyte

It has become clear that the rejection of allograft is due to an immunological reaction (5) and the cells involved in this reaction are the peripheral, small lymphocytes which previously had been thought to have no such ability, hence a great importance (6—13) is now being placed on immunologically competent cells.

The application of the blastformation phenomenon by lymphocytes to tissue typing began with the work by Novell et al. (14) (1960) in which they used human peripheral leucocytes, and by the observations of Carstain (15) (1962) on the blastformation and mitosis in their tissue cultures of peripheral lymphocytes in the presence of phytohemagglutinin (PHA). Subsequently, Bach et al. (16) have demonstrated that, when peripheral lymphocytes of two irrelevant human individuals are mixed and cultured in the presence of PHA under sterile conditions there also occur blastformation and mitosis, and state that the rate of this blastformation represents the histocompatibility factors between them, hence this mixed culture method is applicable to tissue typing in the tissue transplantation just as erythrocyte typing. Since then the mixed cultures of lymphocytes have been studied extensively, and it has been verified (17—20) that the blastformation phenomenon observable in such instances constitutes the basis of immunology. Further, experiments conducted between identical twins or between two unrelated individuals have also demonstrated (21) that the rate of blast-like cell formation can tell the degrees of the differences in histocompatibility.

On the other hand, by immuno-genetical studies on inbred mice it has been clarified that there are histocompatibility antigens such as H-1 to H-13 , and H-2 antigen which is the strongest antigen among them is most intensively being studied. Snell et al. (22) demonstrated practically all the H-2 antigens in the inbred mice. Following the reports by Fikrig (23) and Dutton (24), with rabbits or inbred mice the rate of blastformation in the cultures of lymph-node cells is somowhat lower, but there can be observed blastformation phenomenon just as in the case with peripheral blood lymphocytes. Tanaka et al. (1, 2) have shown that the rate of blastformation in the mixed cultures of lymph-node cells can quite accurately tell the differences in H-2 antigens of several inbred mice whose locus of H-2 antigens differs. In view of this, for the purpose to clarify whether or not the blastformation in the mixed culture can tell the intracellular localization of histocompatibility antigens of lymph-node cells, mixed cultures

were carried out with the homogenate of one group of lymph-node cells previously destroyed by mechanical treatment and other group of the similar live lymph-node cells and studied the relationship of H-2 antigen difference to the rate of blastformation. As a result it has been clarified that there is a significant correlation between the differences in H-2 antigens and the rate of blastformation. Therefore, such lymph-node cell homogenate was further fractionated by centrifugation into subcellular fractions and mixed them with live lymph-node cells, and the rate of blastformation of lymphocytes in the presence of each subcellular fraction was observed.

PHA (phytohemagglutinin)

PHA has originally been extracted from *Phaseosus vulgaris* for the purpose to separate and collect leucocytes from whole peripheral blood, but since it has been shown that this substance has the properties to induce DNA synthesis and mitosis of lymphocytes by later studies (14), it has come to be extensively used in the mixed cultures of leucocytes and lymphocytes. At present there are two kinds of such commercial preparations; PHA-M and PHA-P which is 100-fold stronger than the former, both sold by Difco Company.

As for the concentration of PHA used in the mixed lymphocyte cultures, PHA-M is generally used in the concentration of 1 % (v/v), but these is as yet no accurate data on it. KANEDA (3) states that he found 1 % (v/v) PHA-M to be the optimal concentration in mixed lymph-node cell cultures after trying the concentrations of 0.3 % to 10 % (v/v), and the same is true with the mixed cultures with cell homogenate.

Methods and results

The cells used in the present experiments were lymph-node cells derived from cervical and axillary lymph nodes of inbred mice, and 98 % of the cells were small lymphocytes. HANAOKA (5) also has reported similar results by his phase-contrast microscopic and electron microscopic observations, and the cells appeared at 48 hours later in the present experiments are small lymphocytes.

This mixed culture of lymph-node cell homogenate with live lymph-node cells is the so-called one way stimulation method, where one cell group is placed in the state that their antigens only can react. For such one way stimulation test there are such methods as the use of ^{60}Co (1, 2), the method using of Mitomycin C (25), and the freezing-thawing method (16), and by all these methods only the reaction of one group of lymphocytes to the antigens of the other group can be observed. However, there

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is no report of the mixed cultures with subcellular fractions of the one cell group destroyed mechanically.

Looking at the mixed culture groups with homogenate in the presence of PHA, the rate of blastformation is high in those groups of lymph-node cell homogenate of A or C57BL mice with live lymph-node cells of CBA mice where the differences in H-2 antigens is major, whereas in the combinations of mixed cultures where the H-2 antigen difference is minor, none, or the combination of the same cells, the rate of blastformation shows no significant difference from the respective controls. In the groups without addition of PHA, differing from the mixed cultures between the two live cell groups (1, 2) the blastformation significantly reflects the difference in H-2 antigens. This is similar to the results of the mixed cultures among live cells in that, when the homogenate of those cells with a greater number of different H-2 antigens is added as the antigen, a higher rate of blastformation can be observed, especially in the group added with PHA only those with major differences in H-2 antigens can be identified, indicating the presence of antigenicity in the homogenate.

According to HAUGHTON (26), H-2 antigen activity of homogenized lymph node cells is about 80 % of the total H-2 antigen titer of the cells, and when this homogenate is in contact with antibodies, there occurs more rapid and more complete antigen-antibody reaction because three-dimensional interference is less, differing from the contact with live cells (or unbroken dead cells). This result is supported by POST et al. (27).

This point can explain convincingly the alterations in the rate of blastformation as observed in the present experiment. In contrast, to the mixed cultures of live cells only, where the peak of blastformation was observed 4 to 8 days of the culture as reported by BACH F. et al. (16), BACH, F. H. et al. (25), NOVELL (14), and FIKRIG et al. (23), in the mixed cultures with cell homogenate, ultracentrifuged sediment fractions, and homogenate of the cells destroyed by supersonication to be described in Report 2 (46), the reaction time is short and a considerable blastformation can be observed already at 24-hour culture, and the maximum is reached by the 48-hour culture. In addition, the fact that the rate of blastformation in the groups without PHA can demonstrate significant differences in H-2 antigens, the like of which cannot be observed in the mixed cultures of different live lymph-node cells, might be interpreted as due to a very rapid and complete adhesion of the subcellular fraction on the entire surface of the target cells to elicit more swift blastformation than in the mixed culture of live lymph-node cells. This point can be understood from the result that the time of maximum blastformation of single cell cultures

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with PHA is at the culture day 4 as reported by TANAKA *et al.* (1, 2).

Next, in order to locate the strongest antigenic potency in the cell homogenate with antigenicity, a study was made by SCHNEIDER's liver cell fractionation method (28, 29) with lymph-node cells of mice. In this instance, the localization of antigenic potency was also determined by the blastformation in the mixed cultures of live lymph-node cells with each subcellular fraction. The live cells were mixed with each fraction in the ratio of 1 : 4 because the final recovery rate of each fraction proved to be 1/4 the volume of the live cell. As already described, it has become clear that the highest antigenic potency is in the microsomal fraction, followed by the mitochondrial fraction. Even the nuclear fraction showed some antigenic activity but this is supposed to be due to the contamination of the two fractions mentioned above.

There are many such studies on the localization of the antigenic potential in the ultracentrifuged fractions. As materials liver and spleen were used, and most of the studies determine the localization of antigens by the survival time (or the shortening of the survival) of the skin trans-plants on the animals after the administration of such centrifuged frac-tions. Namely, AL-ASKARI *et al.* (30) prepared homogenates of liver and spleen of mice and fractionated these homogenates in 0.25 M sucrose solution by centrifuging at 105, 000 × g, and in the skin transplantation to the mice sensitized with these fractions found the microsomal fraction to contain the most potent antigens by the degree of rejection of the skin transplants. They reported also species specificity in these fractions. Aside from these, there are reports (31—37) supporting the idea of transplanta-tion antigenicity located in the microsomal fraction. On the other hand, BASCH *et al.* (38), by the erythrocyte agglutination inhibition tests and the cytotoxicity method and later AL-ASKARI (39), by observing the rejection of skin transplants in the mice after the injection of mouse spleen mito-chondrial fraction, found the mitochondrial fraction to contain strong histocompatibility antigens, and also LAWRENCE (40), and MONACO *et al.* (41) made similar reports. There are also reports (42, 43) contending that such antigens are contained in the fraction intermediate between the microsomal and the mitochondrial fractions.

Although results differ according to the authors as already stated, recently, it seems that such transplantation antigens are localized on the cell surface and in the membraneous components of intracellular organellae corresponding to the microsomal and the mitochondrial fractions prepared by centrifugation (44) as reported by DAVIES (45, 46) in his successful extraction of soluble antigens from the cell membrane.

Looking at the results of the present experiments from the aspect of the difference in H-2 antigens, distinct differences of H-2 antigens can be observed more clearly in the $8,500 \times g$ and $100,000 \times g$ fractions than in the case with homogenates. Hence, when the cells of such strain that have many H-2 antigens which the recipient, live lymph-node cells do not have, are made to act as donor antigens, the rate of blastformation becomes marked, and when the cells with minor difference or no difference in H-2 antigens are made to act on the target cells, the rate of blastformation is low or shows hardly any difference from that of respective controls, proving that the rate of blastformation in such cultures can tell definitely the magnitude of differences in H-2 antigens. In other words, in the combination of A which has 8 different H-2 antigens against C57BL and C57BL, or in the combination of A or C57BL having 8 different H-2 antigens against CBA and CBA, when the $8,500 \times g$ or the $100,000 \times g$ sediment fraction of the former cells is added to the latter living cells of each combination, there can be observed a marked blastformation, while, on the contrary, in the mixed culture of AKR (sediment fraction)+A (live cells) where there is one different H-2 antigen, and in the combination of AKR (sediment fraction)+CBA (live cells), the rate of blastformation is as low as to show no difference from that of the controls. These results indicate that there is a close correlation between the rate of blastformation of this experiment using sediment fraction and those of mixed cultures using both live cells in which each of the two group cells reacts as antigen and antibody (2). The reason why the rates of blastformation in the mixed cultures of the $100,000 \times g$ sediment fractions+live lymph-node cells are higher than that in the mixed cultures of both live cells seems to lie in the fact that such sediment fractions are closer to pure antigen component, and they have better adhesive abilities, as pointed out by HAUGHTON. Aside from this point, the fact that the rate of blastformation differs by the kind of mixed cultures in spite of the same number of different H-2 antigens might be interpreted as due to differences in the antigens other than H-2 antigens.

CONCLUSION

1. The cells used in the present experiments were lymph-node cells from inbred mice, and over 98 % cells were proven to be small lymphocytes. Therefore, those cells that have undergone blastformation are all those derived from small lymphocytes.

2. When homogenate of one cell group is cultured with live cells of the other pairing group, there occurs blastformation. In the presence of

PHA, such a blastformation becomes more marked.

3. The optimal concentration of PHA (phytohemagglutinin)-M added to the mixed culture is found to be 1 % (v/v).

4. The maximum rate of blastformation in the mixed culture is observed at the culture hour 48, being much faster than in the mixed culture between two live cell groups.

5. In the mixed cultures between subcellular fractions prepared from cell homogenate by centrifugation and live cells, the transplantation antigenic potential (histocompatibility antigenic potential) is seen in the mitochondrial and the microsomal fractions, especially marked in the latter.

6. In the observations carried out by various combinations of these inbred mice, it has been demonstrated that the rate of blastformation induced by the addition of cell homogenate or sediment fractions prepared from the homogenate reflects quite accurately the differences in H-2 antigens.

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Photograph
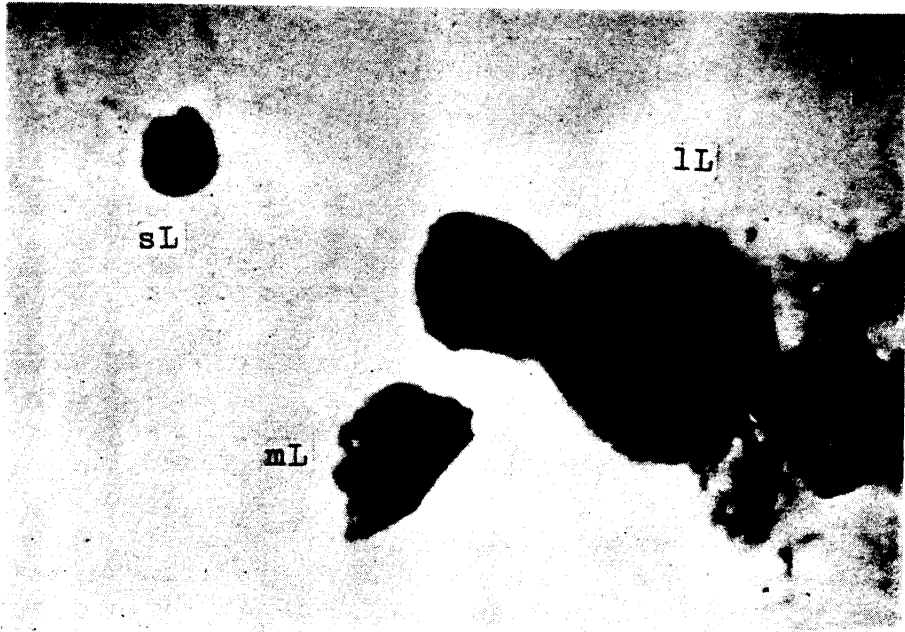
This is a picture of the smear specimen of the cells in mixed culture of the sediment from mouse lymphocyte homogenate plus homologous live lymphocytes added with 1 % (v/v) PHA-M prepared at culture hour 48. There can be observed large, intermediate and small lymphocytes ($\times$ 1000). Intermediate lymphocyte (mL) and large lymphocyte (lL) are taken as blast-like cells.