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Tissue typing by mixed culture of lymphocytes. II. Demonstration of H-2 antigen differences by mixed cultures with addition of subcellular fractions prepared from homogenate of lymph-node cells destroyed by supersonication

Hiroaki Miwa*

*Okayama University,

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Tissue typing by mixed culture of lymphocytes. II. Demonstration of H-2 antigen differences by mixed cultures with addition of subcellular fractions prepared from homogenate of lymph-node cells destroyed by supersonication*

Hiroaki Miwa

Abstract

1. It has been found that mouse lymph-node cells, even destroyed by sonication with 20 KC supersonicator, maintain sufficient antigenicity both in vitro and in vivo. 2. When such sonicated cell homogenate is cultured with live lymph-node cells, there can be observed blastformation and the peak of the rate of the blastformation is seen at culture hour 48. 3. When PHA (phytohemagglutinin)-M is added to such mixed cultures, the blastformation is enhanced. 4. When mixed cultures of mouse lymph-node cells are conducted by using such one-way stimulation method in various combinations, the rate of blastformation can tell quite accurately the differences in H-2 antigens of mice. 5. In the experiment using F1 hybrid mice and the parents, it has been demonstrated that the rate of blastformation in mixed cultures of the present experiments shows a direct correlation to the rate of blast formation in mixed cultures of live lymph node cells, while it is an inverse proportion to the survival time of the skin transplant. 6. Differences in the transplanation antigens said to be located on sex chromosomes cannot be distinguished by this one.way stimulation method.

**TISSUE TYPING BY MIXED CULTURE OF LYMPHOCYTES
II. DEMONSTRATION OF H-2 ANTIGEN DIFFERENCES BY
MIXED CULTURES WITH ADDITION OF SUBCELLULAR
FRACTIONS PREPARED FROM HOMOGENATE OF LYMPH-
NODE CELLS DESTROYED BY SUPERSONICATION**

Hiroaki MIWA

*Department of Surgery, Okayama University Medical School, Okayama, Japan
(Director : Prof. S. Tanaka)*

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In the previous report (31) it was stated that in the mixed cultures of live lymph-node cells having known H-2 antigens with addition of subcellular components of lymph-node cell homogenate these live cells showed marked blastformation by which the differences in H-2 antigens could be distinguished clearly. However, since the procedures of cell destruction are rather complicated, it remains uncertain whether or not transplantation antigens were present in the components of the cells so destroyed. Therefore, in the present experiments cells were destroyed by a simple method of supersonication, and it has been observed that subcellular fractions prepared from the cells so destroyed induce blastformation and transplantation immunity in live lymphnode cells, which enables us to differentiate accurately the differences in H-2 antigens.

MATERIALS AND METHODS

Materials : Inbred mice used were A (H-2^a), C57BL (H-2^b), C3H (H-2^k), CBA (H-2^k) and AKR (H-2^k), all being 2—3 months old, and obtained from the Mouse Colony of Okayama University Medical School. In addition, F₁ mice, a cross between A (male) and C3H (female) of the above group, were used, also 2—3 months old.

And the preparation of culture medium, antibiotics and PHA-M (Bacto-phytohemagglutinin-M, Difco) were the same as described in Report 1 (31).

For supersonication Model T-A-4201 apparatus of Kaijo Electric K. K. was used. The tips were always of 7 ϕ tip (7 mm in tip diameter), and the vessels for destruction of lymph-node cells were 8 ml-centrifugal polyethylene tubes.

Methods : The procedures of preparation of cell samples are the same as in the previous report (Report 1).

Two ml of the live lymph-node cell suspension adjusted to 10⁷ cells/ml is transferred into 8 ml-centrifugal polyethylene tube, and with the supersonication

apparatus the tip end of which is dipped into the suspension 0.5 cm deep and fixed there, while cooling the tube in the ice-bath, the sonication is conducted. The cell homogenate so prepared by the supersonication is taken as the homogenate corresponding to 10^7 cells/ml of lymph-node cells.

The composition of medium, concentration of PHA-M and antibiotics are used as described in Report 1.

To the medium is added the mixture of 10^6 cells/ml of live lymph-node cells and the supersonicated homogenate in the ratio of 1:1 (v/v). Nine tubes each of the mixed culture solution prepared in the above fashion are made each time, cultured in an incubator at 37°C, for 24, 48, 72, some times 96 hours and further up to 7 days. After respective culture time the tubes are taken out, centrifuged at 800 rpm for 10 minutes, and the cells in the sediment are resuspended in the remnant solution with micropipette by gentle stirring. A droplet of this cell suspension is smeared on several slideglasses, and the smears are stained with May-Grünwald-Giemsa stain.

The standard for determining the size of lymphocytes by microscopy is large $> 110 \mu^2$, $56 \mu^2 < \text{intermediate} < 110 \mu^2$ and small $< 56 \mu^2$ (1, 2, 3).

1. Conditions of lymph-node cell destruction

i) *Optimal conditions in vitro*: For the purpose to determine the extent of lymph-node cell destruction and the presence of antigenicity, the conditions for supersonication are fixed as in the foregoing. The lengths of the sonication time are set at 30", 60", 90", 120", 180", 240", and 300", repeating each experiment three times. The optimal conditions of the cell destruction are determined by the extent of cell destruction observed by microscopy at each time and also by the rate of blast-like cell formation in the mixed culture of A supersonicated lymph-node cell homogenate + CBA live lymph-node cells.

ii) *Study on the antigenicity in vivo*: C3H mice are sensitized by inoculating subcutaneously on the back sonicated A lymph-node cell homogenate in the amount corresponding to 10^7 cells, and 7 days later the skins from A mice are transplanted onto the back of the sensitized C3H mice to see the manner of rejection of the skin transplants. For the control the skins of A mice are transplanted to untreated C3H mice. In the skin transplantation C3H mice are anesthetized by subcutaneous injection of 0.001 ml/g Nembutal undiluted solution, hair on the back of the animal is denuded extensively, then the circular area in the middle of the back is denuded of the skin, the skin graft from A mouse of the whole thickness is transplanted on the back, fixed with Alon alpha, covered with Aeroplast, and Band-aid (Johnson & Johnson) is bandaged over it. This Band-aid is removed 3 days later, and thereafter daily observations are carried out until the complete rejection of the transplant.

2. Changes in the number of the cells with lapse of culture time

The observation method of single cell culture was as already reported (31) (original number 10^6 cells/ml, cultured in the medium composed of HANKS solution, YLE, calf serum, and TC-199 solution, 1:2:2:5, (v/v)). In the present experiment concurrently with the single cultures, mixed cultures of 10^6 cells/ml

live lymph-node cells with supersonicated cell homogenate (1 : 1, v/v) with or without PHA are conducted, and the changes in the cell number are observed with lapse of time.

3. *Changes in the rate of blastformation*

Changes in the rate of blastformation in the mixed cultures of homogenized cell sediment fractions with live lymph-node cells were mentioned in Report 1. Such changes are also observed in the present experiment of mixed cultures with supersonicated cell homogenates in various combinations.

4. *Single cell culture groups*

The single cell cultures are conducted by adding live lymph node cells only to the medium with or without PHA-M to see the rate of blast like cell appearance at culture hour 48 as the control experiments for respective mixed culture groups conducted under similar conditions.

5. *Mixed culture groups*

i) *Combination of the same sex*: Combinations of males or females of the allogeneic mice are variously tried, and one of the two cell groups is previously destroyed by supersonation, which is then mixed with live cells of the other group, and cultured. The combinations used were 4 sets each between CBA, A, and C57BL (where the difference of H-2 antigens is present), 2 combinations between CBA, and AKR (where the H-2 antigen difference is absent), and further the iso-combinations of A themselves and CBA themselves to the total of 10 sets of combination.

ii) *Combination of F_1 with parent cells (F_1 -test)*: Four combinations are prepared between (A ♂ × C3H ♀) F_1 , A, and C3H cells, all obtained from mice 2—3 months old.

iii) *Combination of male and female*: Two combinations each were made between male and female of C57BL and C3H.

RESULTS

1. *Optimal conditions for supersonation to destroy lymphocytes*

i) *The optimal conditions in vitro*: The results are given in Fig. 1 of the observations on the smear specimens prepared after the 48-hour culture of the combinations (CBA live lymph-node cells + sonicated A cell homogenate) with or without PHA, what appeared to show the most marked blastformation for the purpose to study the problems of the time required to destroy the cells and the antigenicity.

On looking at the relationship between the time of destruction of the cells and the rate of blastformation, in the groups added with PHA the maximum rate of blastformation was 29.9 % by 2 minute sonication, and also even in the groups without PHA, although some of them were not

tested, the maximum rate of blastformation was 22.5 % after the 2-minute sonication. In the microscopic observation of the smear specimen of the supersonicated homogenates, shorter than 90", there could be seen some live cells, but with the smears prepared after longer than 2-minute sonication, the cells were destroyed uniformly, leaving no live cells.

From these findings it is thought that the 2-minute supersonation is the optimal length to destroy lymph-node cells as well as to obtain the maximum rate of blastformation in the mixed culture under the conditions mentioned in the foregoing.

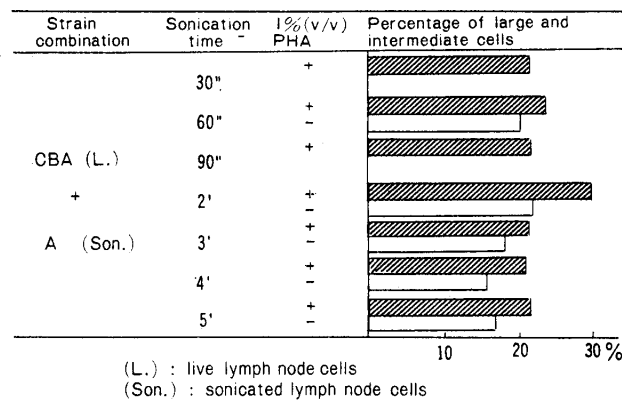


Fig. 1 Relationship between sonication time and the rate of blastformation

ii) *Study on the antigenicity in vivo of supersonicated cell homogenate* : After transplanting the A skin graft of whole thickness on the back of C3H mice previously sensitized with sonicated A lymph-node cell homogenate equivalent to contain to 10^7 lymph-node cells, the rejection of the skin transplant was observed macroscopically with lapse of time. The results are as shown in Fig. 2. As for the criteria of determining the rejection, besides from edema, hemorrhage, reddening and the spread of necrosis, diagnostic findings such as induration and infiltration were taken into consideration, and the time when hemorrhagic spots, necrosis and induration spread over 50 % of the transplant was considered to be the median survival time (4). While the survival time of the control (A skin to untreated C3H mouse) was 11.4 ± 1.5 days, it was 7.4 ± 0.8 days in the test groups sensitized with sonicated cell homogenate, showing the shortening of survival, which proves that transplantation antigenicity against the sonicated cell homogenate remains clearly intact.

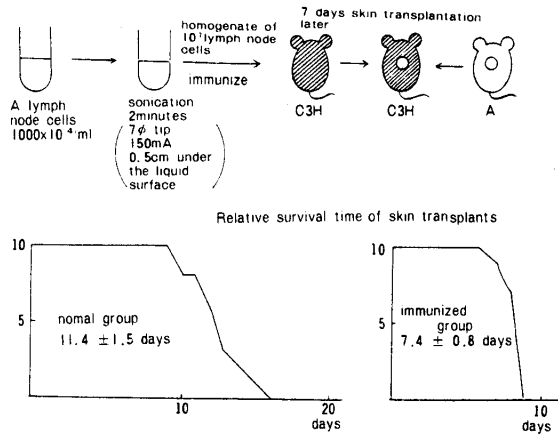


Fig. 2 Diagrammatic drawing of sensitization and skin transplantation

2. Changes in the cell number in tissue culture with lapse of time

Regarding the changes in the cell number during tissue culture using the medium described, a report was made in the previous paper (31), but the results of the present experiments coincide with those reported already. It has been found that by the 48-hour culture time when the blastformation reaches its maximum, about 80% of the cells have still survived. In the observations on the number of cells with the groups of live lymph-node cells plus supersonicated cell homogenate both with and without PHA, the results proved to be approximately the same as those of single live cell groups. Up to the culture day 7 about 70% of the cells survive, indicating that by the methods of tissue culture employed in the present experiment changes in the cell number can be sufficiently observed up to 7 days.

3. Changes in the rate of blastformation

The cells observable at the cell counting prior to tissue culture on the whole are small cells of about the same size over 98% of the total cells being small lymphocytes. These observations are similar to those of the smear specimens as in Report 1.

The changes observable in the blastformation of the mixed culture in the combination of these live cells with sonicated cell homogenate of other strain of mice in the ratio of 1 to 1, with 1% (v/v) PHA are as shown in Fig. 3. In the case of C3H live lymph-node cells plus sonicated A lymph-node cell homogenate (1 : 1) the rate of blastformation can be seen in 20% of the cells at culture hour 24, it is 27.9% by culture hour 48, which is the maximum, and by 72-hour culture, contrary to the expectation, it decreases to 18.3%, thereafter it decreases along with lapse of time, fall-

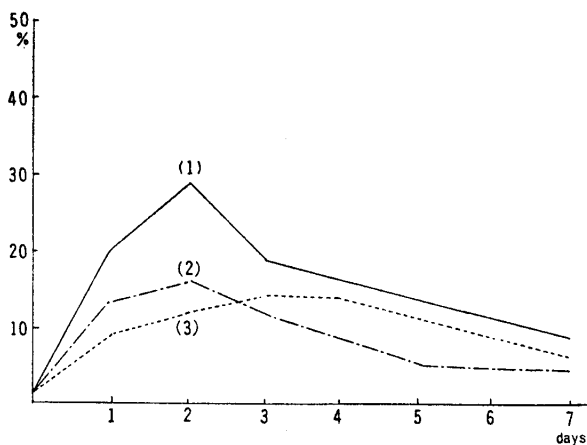


Fig. 3 Changes in the rate of blastformation with lapse of time in mixed cultures of sonicated cells and untreated allogeneic cells

(1): C3H (L.) + A (Son.) with 1% (v/v) PHA-M

(2): C57BL (L.) + C57BL (Son.) with 1% (v/v) PHA-M

(3): C3H (L.) with 1% (v/v) PHA-M

(L.) : live lymph node cells

(Son.): sonicated lymph node cells

ing down to 8.0% by the culture day 7. This tendency can be observed similarly both in the combination of strains without any difference in H-2 antigens and in the same strain combination, and the maximum blastformation can also be seen at culture hour 48, though this rate is somewhat lower but the differences in H-2 antigens are most marked at this stage. In contrast to this, in the single lymph-node cells culture with PHA, differing from the former groups, the maximum rate is seen slightly delayed, between culture hour 72 and 96, thereafter it tends to decrease.

These results suggest that for the purpose to see the difference in the rate of blastformation of mixed cultures added with sonicated cell homogenate it would be appropriate to compare the results at culture hour 48.

4. Single cell culture groups

On observing the rate of blastformation in the single lymph-node cell group with addition of 1% (v/v) PHA and the single group without PHA, the rate in the group with 1% PHA is 12.2% and it is 7.9% in the one without PHA (Tables 1 and 2).

Table 1 Percentage of large and intermediate cells in single cell cultures (CBA lymph node cells)

Experiment No.	Percentage without PHA-M	Percentage with 1% PHA-M
1	9.4	13.6
2	9.0	13.0
3	8.0	12.5
4	9.0	11.5
5	9.4	10.8
6	6.5	15.0
7	7.4	11.6
8	7.5	12.0
9	5.8	10.6
10	7.0	11.3
Average	7.9	12.2 ($p < 0.001$)

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

Strain	Number of times of exp's.	Percentage without PHA-M	Percentage with 1% (v/v) PHA-M
A	10	7.6	11.9
C57BL	8	7.1	11.7
CBA	31	7.7	12.5
C3H	4	7.2	12.1
AKR	4	5.8	11.4
(A ♂ × C3H ♀) F ₁	4	6.9	12.4
Average		7.1	12.0 ($p < 0.001$)

5. Mixed culture groups

i) Combinations of the same sex between different strains having different H-2 antigens

In the case where the difference in H-2 antigen is major : Referring to the table of H-2 antigenic phenotype of mice (5, Report 1), for the mixed cultures of the supersonicated cell homogenate proven to contain antigenicity and live lymph-node cells, at first the lymph-node cells (ones to be used as donor) having many H-2 antigens were destroyed by supersonation. The results are as shown in Table 3. When A lymph-node cells with 8 different H-2 antigens from CBA are mixed with live CBA lymph-node cells in the presence of 1% (v/v) PHA, the rate of blastformation is 28.9% in average, while the mixed cultures without PHA show blastformation

Table 3 Percentage of large and intermediate cells in mixed cultures of sonicated cells and untreated allogeneic cells
H-2 antigen difference

Strain combination	Number of times of exp's.	Percentage without PHA-M	Percentage with 1% (v/v) PHA-M
CBA (L.) + A (Son.)	15	22.2 ($p < 0.001$)	28.9 ($p < 0.001$)
CBA (L.) + C57BL (Son.)	7	22.9 ($p < 0.001$)	26.6 ($p < 0.001$)
A (L.) + CBA (Son.)	5	10.8 ($p < 0.001$)	14.8 ($0.05 < p < 0.1$)
C57BL (L.) + CBA (Son.)	4	13.0 ($0.01 < p < 0.02$)	16.0 ($0.001 < p < 0.1$)

(L.) : live lymph node cells

(Son.): sonicated lymph node cells

in 22.2% of the cells. In the mixed cultures of C57BL (sonicated homogenate) having also 8 different H-2 antigens to CBA + CBA (live lymph node cells), the rate of blastformation is 26.6% in the groups added with PHA and 22.9% in those without PHA.

In the case where the difference in H-2 antigence is minor : On the other hand, in the reverse combinations, namely, CBA (sonicated homogenate) with one different H-2 antigen to A + A (live cells) the groups with PHA shows blastformation in 14.8% while the group without PHA 10.8% ; and in the combinations of CBA (sonicated homogenate, also with 6 different H-2 antigens to C57BL)+C57BL (live cells), the groups with PHA shows the blastformation in 16.0% whereas the groups without PHA as low as 13.0%.

ii) *Combinations of the same sex between different strains but no difference in H-2 antigens :* By the combinations without difference in H-2 antigens such as CBA, AKR in the combinations of CBA (live cells) + AKR (sonicated homogenate) the groups added with PHA show the blastformation in 13.0% , while the groups without PHA 9.7% . In the combinations of AKR (live cells) + CBA (sonicated homogenate) the groups with PHA show 11.5% blastformation, but the groups without PHA as low as 9.2% (Table 4).

iii) *Combinations of the same sex between the same strains :* The results of the iso-combinations are as illustrated in Table 4. In the combinations of A cells themselves the group with PHA shows 12.0% blastformation and the groups without PHA 9.6% , while in the groups of CBA cells themselves the one with PHA 12.3% and the one without PHA 9.6% . In the groups without any H-2 difference and in the groups of the same strains, the groups added with PHA show less than 15% blastformation.

Table 4 Percentage of large and intermediate cells in mixed cultures of sonicated cells and untreated allogeneic cells
Without difference in H-2 antigen

Stain combination	Number of times of exp's.	Percentage without PHA-M	Percentage with 1% (v/v) PHA-M
CBA (L.) + AKR (Son.)	4	9.7 (0.01 < p < 0.02)	13.0 (0.01 < p < 0.02)
AKR (L.) + CBA (Son.)	4	9.2 (0.01 < p < 0.02)	11.5 (0.8 < p < 0.9)
A (L.) + A (Son.)	5	9.6 (0.001 < p < 0.01)	12.0 (0.2 < p < 0.3)
CBA (L.) + CBA (Son.)	5	9.6 (0.02 < p < 0.05)	12.3 (0.2 < p < 0.3)
CBA (L.)	31	7.7	12.5 (p < 0.001)

(L.) : live lymph node cells

(Son.): sonicated lymph node cells

Table 5 Percentage of large and intermediate cells in mixed cultures of sonicated cells and untreated allogeneic cells
F₁ test (A ♂ × C3H ♀) F₁

Strain combination	Number of times of exp's.	Percentage without PHA-M	Percentage with 1% (v/v) PHA-M
C3H (L.) + F ₁ (Son.)	4	14.2 (p < 0.001)	19.9 (p < 0.001)
A (L.) + F ₁ (Son.)	4	13.2 (0.001 < p < 0.01)	15.2 (0.02 < p < 0.05)
F ₁ (L.) + C3H (Son.)	4	12.5 (p < 0.001)	14.1 (0.02 < p < 0.05)
F ₁ (L.) + A (Son.)	4	10.1 (0.6 < p < 0.7)	12.1 (0.7 < p < 0.8)
F ₁ (L.)	4	6.9	12.4 (p < 0.001)

(L.) : live lymph node cells

(Son.): sonicated lymph node cells

iv) *F₁-test* : The results of this test are as shown in Table 5. H-2 difference of F₁ to C3H (female) corresponds to 8/2, and in the combinations of C3H (live cells) + F₁ (sonicated homogenate), the groups with PHA show 19.9% blastformation while the groups without PHA 14.2%. In contrast, in the reverse combinations of F₁ (live cells) whose H-2 difference corresponds to 0 + C3H (sonicated homogenate), the one with PHA and the other without PHA show blastformation in 14.1% and 12.5% of them, respectively. Further, in the combinations of A (live cells) with F₁ (sonicated homogenate), the combination of A (live cells) whose H-2 difference

corresponds to $1/2 + F_1$ (sonicated homogenate) shows 15.2% of blastformation in the presence of PHA but 13.2% without PHA, whereas the combinations of F_1 (live cells) + A (sonicated homogenate) 12.1% in the presence of PHA and 10.1% without PHA.

v) *Combinations of male with female of the same strains* : The combinations thus far described were either male against male or female against female in order to eliminate sex differences, but in this experiment alone male and female combinations were attempted (Table 6).

Table 6 Percentage of large and intermediate cells in mixed cultures of sonicated cells and untreated allogeneic cells
Sex linked histocompatibility antigen

Strain combination	Number of times of exp's.	Percentage without PHA-M	Percentage with 1% (v/v) PHA-M
C57BL ♂ (L.) + C57BL ♀ (Son.)	4	9.5 ($0.02 < p < 0.05$)	13.5 ($0.1 < p < 0.2$)
C57BL ♀ (L.) + C57BL ♂ (Son.)	4	9.5 ($0.01 < p < 0.02$)	13.7 ($0.2 < p < 0.3$)
C3H ♂ (L.) + C3H ♀ (Son.)	4	7.1 ($p > 0.9$)	11.3 ($0.1 < p < 0.2$)
C3H ♀ (L.) + C3H ♂ (Son.)	4	6.7 ($0.001 < p < 0.01$)	13.4 ($0.05 < p < 0.1$)

(L.) : live lymph node cells

(Son.) : sonicated lymph node cells

With C57BL, in the combination of male (live cells) + female (sonicated homogenate) the groups with PHA showed blastformation in 13.5% and the one without PHA in 9.5%, while in the reverse combinations of female (live cells) + male (sonicated homogenate) the groups with PHA showed blastformation in 13.7% of the cells and the one without PHA in 9.5%.

And with C3H, in the combination of male (live cells) + female (sonicated homogenate) the groups with PHA showed blastformation in 11.3% and the one without PHA in 7.1%, while in the reverse combinations of female (live cells) + male (sonicated homogenate) the groups with PHA show blastformation in 13.4% of the cells and the one without PHA in 6.7%.

All these values are the average of at least 4 trials or more each.

DISCUSSION

There are reports (6, 7) stating that in peripheral blood lymphocytes cultured for several days in the presence of PHA (phytohemagglutinin)

there occur DNA synthesis and mitosis of the cells in culture, and further similar changes can be observed when peripheral blood lymphocytes of unrelated individuals are mixed and cultured, and it is said that the mode of such changes might be applicable to the histocompatibility test (tissue typing) between two such unrelated individuals as reported by BAIN *et al.* (8) and BACH *et al.* (9). Since then there appeared many reports (10, 11, 12) concerning the blastformation of lymphocytes in culture using peripheral leucocytes. It is known that DNA synthesis and mitosis can be observed in the tissue cultures of animal peripheral blood lymphocytes and lymphoid cells, as demonstrated by FITZGERALD *et al.* (13, 14); and DOWD *et al.* (15) in their tissue cultures of peripheral blood lymphocytes, and by RIEKE *et al.* (16) with peripheral blood lymphocytes and thymus lymphoid cells, and by DUTTON (17) with spleen and lymphoid cells, especially noteworthy is the report of DUTTON *et al.* in which they observed DNA synthesis in the mixed cultures of lymph-node cells of two different groups using radio-isotope.

It was FIKRIG (18, 1966) who first reported about blastformation using rabbit lymph-node cells just as in the present experiments, in which they cultured rabbit lymph-node cells in the presence of PHA for 5 days and found similar blastformation, though its rate was low. Further, in 1967 TANAKA *et al.* (1, 2) reported for the first time that the rate of blast-like cell formation can tell quite accurately H-2 differences, and emphasized the importance of correlation between the difference in histocompatibility factors and blastformation in their mixed cultures of mouse lymph-node cells with PHA. However, since such mixed cultures are done between two groups of live cells, it has a disadvantage in knowing which group of the cells reacted more because the interreaction occurring would be the sum total reactions of the two groups. As a solution to this problem BACH *et al.* (11) performed such mixed cultures by repeated freezing-thawing treatment of one of the two groups before the culture, and by exposing one group of cells to Mitomycin-C before the culture (19). TANAKA *et al.* (1, 2) carried out the mixed culture after exposing one group of cell to ^{60}Co before the culture. As reported in the previous paper (31), on the basis of the fact that there occurs blastformation even when one group of lymph-node cells are destroyed mechanically and cultured with the other group of live cells, and the rate of such blastformation can distinguish quite accurately H-2 difference, the present experiments were conducted with cells destroyed by supersonication which yields rapid and uniform cell destruction.

The fact that lymph-node cells destroyed by supersonication still retain antigenicity is demonstrated by *in vitro* experiments such as the mixed

cultures of CBA (live cells) + A (sonicated homogenate) in the presence of PHA in all the experiments blastformation was observed in more than 20% of the cells, as compared with 12.2% in the controls (single cell cultures) and more than 15% of the groups without PHA, as compared with 7.9% in the controls, showing the accelerate blastformation by a significant difference. This fact is also demonstrated by such *in vivo* experiments as when the mice are sensitized with such sonicated cell homogenate at the optimal time of 2-minute sonication and skin graft is transplanted 7 days later, there occurs an accelerated rejection of the skin transplant in the sensitized animals. DAVIES (20, 21), HANAOKA (22) and BRENT (23) also demonstrated similar phenomena.

When supersonicated cell homogenate prepared at 2-minute sonication which retains antigenicity as proven *in vitro* and *in vivo* is cultured with live lymph-node cells and the survival of the cells is observed, the results are similar to those of the mixed cultures of centrifuged sediment + live cells as previously reported, and the rate of blastformation likewise reaches its maximum at culture hour 48. This seems to be due to the fact that by supersonation the cell components are broken into more minute particulates so that these minutes particle can attach themselves onto the entire surface of target cells to react more rapidly and this fact can be also understood from the theory presented by HAUGHTON (24) and POST *et al.* (25).

Considering the rate of blastformation and difference in the transplantation H-2 antigens the rate of blastformation is seen in almost 30% of the cells with PHA in the combinations which have many H-2 differences, and it is as high as 20% in the groups even without PHA. In contrast, the combinations with less difference in H-2 antigens the groups with PHA show a low blastformation in a little over 15% of the cells, in the combinations without difference in H-2 antigen the blastformation is about 12% which is close to the control. This is also true with the combinations without PHA, proving that the rate of blastformation faithfully reflects the difference in H-2 antigens which are strong transplantation antigens. Of these combinations, H-2 difference in combination of CBA (sonicated homogenate) + A (live cells) is one, whereas CBA (sonicated homogenate) + C57BL (live cells) has 6 different H-2 antigens, but both show about the same rate of blastformation. These findings raise question whether there is difference in the potency of H-2 antigens or whether there is effect of antigens other than H-2 antigens.

It is interesting to look at the combinations between F_1 which is believed to have $1/2$ antigens of each parent and the each of its parents. F_1 is

thought to possess all the antigenic factors of the parents (26) other than these in chromosomes, which is indispensable to the study of immunology. When skin graft from either one of the parent mice is transplanted to F_1 , it survives permanently, but when the skin graft of F_1 is transplanted to either one of parents, as F_1 possesses antigens of both parents, the skin transplant is rejected by the formation of antibodies induced by the antigens lacking in either one of the parents. The observations on blastformation faithfully reflects this point; namely in the mixed cultures of F_1 (sonicated homogenate) added with C3H (live cells) or A (live cells) the blastformation in the presence of PHA is moderate, being 19.9% and 15.2% respectively, whereas C3H (sonicated homogenate) or A (sonicated homogenate) added to F_1 (live cells) with addition of PHA it is 14.1% and 12.1% which is only comparable to that of respective control, and this fact reveals an inverse correlation with the survival time of the skin transplant. In comparing the results of the mixed cultures of TANAKA *et al.* (1, 2) and those of the present experiments, it is obvious that the result of the present experiments and the rate of blastformation in the mixed cultures of live cells is in a direct proportion, whereas it is in an inverse proportion to the survival time of the skin graft as shown in previous reports (1, 2). It has been reconfirmed that the rate of blastformation indicates quite accurately the differences in H-2 antigens.

In male and female there are X and Y sex chromosomes in addition to autosomes, and there are reports on the histocompatibility antigens related especially to Y chromosomes by EICHWALD *et al.* (27) in the skin transplantation using C57BL and A mice, and these reports (28, 29, 30) are in agreement with the this results. However, in the present experiments with C57BL and C3H mice between male and female I found no significant difference. This seems to be due to the fact that transplantation antigens concerned with sex chromosomes are not so potent as H-2 antigens, so that those antigens other than H-2 cannot be compared with the rate of blastformation.

These findings seem to indicate that the blastformation phenomenon in the mixed cultures of lymph-node cells can tell only the differences in those major transplantation antigens among mice.

CONCLUSION

1. It has been found that mouse lymph-node cells, even destroyed by sonication with 20 KC supersonicator, maintain sufficient antigenicity both *in vitro* and *in vivo*.

2. When such sonicated cell homogenate is cultured with live lymph-node cells, there can be observed blastformation and the peak of the rate of the blastformation is seen at culture hour 48.

3. When PHA (phytohemagglutinin)-M is added to such mixed cultures, the blastformation is enhanced.

4. When mixed cultures of mouse lymph-node cells are conducted by using such one-way stimulation method in various combinations, the rate of blastformation can tell quite accurately the differences in H-2 antigens of mice.

5. In the experiment using F_1 hybrid mice and the parents, it has been demonstrated that the rate of blastformation in mixed cultures of the present experiments shows a direct correlation to the rate of blastformation in mixed cultures of live lymph node cells, while it is an inverse proportion to the survival time of the skin transplant.

6. Differences in the transplantation antigens said to be located on sex chromosomes cannot be distinguished by this one-way stimulation method.

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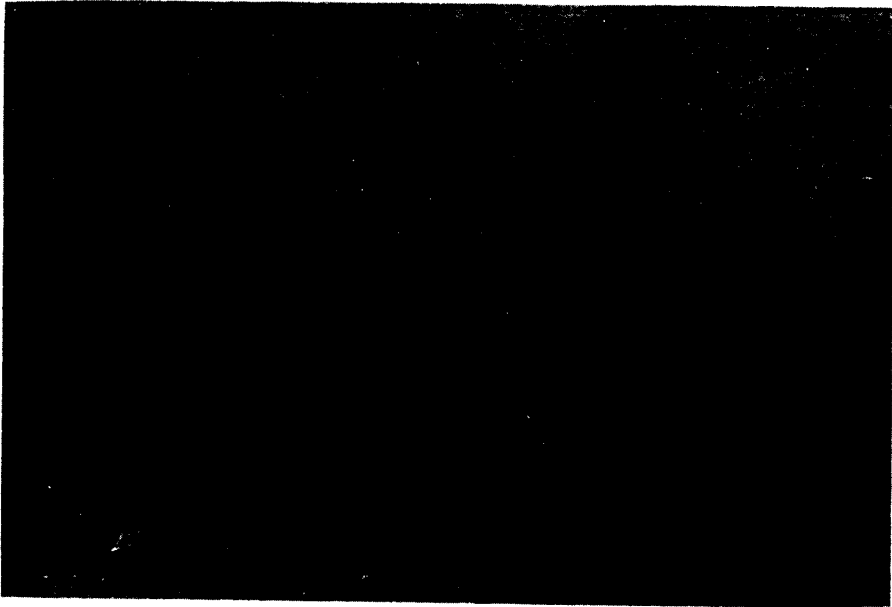
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Photograph



This is a picture of the smear specimen of mouse lymphocyte homogenate (equiv. of 1000×10^4 cells/ml) destroyed by supersonication and stained with May-Grünwald-Giemsa ($1000 \times$). The lymphocytes are destroyed completely showing not any whole cell.