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Received October 12, 1966. P.S.E.B.M., 1967, v124.

Distribution of H-2 Specificities Within the LM Mouse Cell Line and Derived Lines.* (31753)

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Histocompatibility antigens on mouse cells, and antibodies to them, have been demonstrated by a variety of techniques. These include haemagglutination, absorption, cytotoxicity and stimulation of antibody production(1), measurement of colony formation *in vitro*(2), and staining with fluorescent antibody(3).

Gangal, Merchant and Shreffler(1) demonstrated that after 25 years in culture LM cells grown in modified medium 199(4), supplemented with 0.5% peptone (called 199P), still possessed all the specificities of the $H\text{-}2^k$ allele of the C3H mouse from which the L-cell line originally was derived. They demonstrated specificities 1, 3, 5, and 8 by absorption and haemagglutination, and specificity 11 (which was only weakly absorbed) by stimulation of antibody production. As specificity 32 is not readily detected by haemagglutination, they demonstrated it by cytotoxicity.

In the present study an indirect fluorescent antibody test was used to demonstrate all the specificities. The fluorescent antibody test also enables one to check for the presence of antigenic specificities at the cellular level. A proportion of antigen deficient cells in a population would not be observed by absorption tests, but would be detected by the fluorescent antibody method. It was proposed to determine whether all the cells in a population of LM cells carried each of the $H\text{-}2^k$ specificities.

In addition to confirming and enlarging upon the previous results with LM (199P) cells, it was considered interesting to know whether LM cells grown in 2× Eagle basal medium also possessed the known specificities of the $H\text{-}2^k$ allele. Eagle medium is simpler than 199P and is chemically defined(5). LM (2×E) cells are known to differ from LM (199P) cells in their alkaline phosphatase activity as well as in specific chromosome markers and karyotype(6). LM (2×E) cells have no alkaline phosphatase activity but LM (199P) cell populations are composed of a mixture of alkaline phosphatase positive and alkaline phosphatase negative cells(7). It has been suggested that alkaline phosphatase activity is closely associated with H-2 antigen specificity(8). If this were the case, LM (2 × E) cells might be expected to be lacking in one or more of the H-2 specificities.

It was also proposed to check for the presence of H-2 specificities on some other variants of the LM cells, of different karyotype, alkaline phosphatase activity, and colonial and morphological appearance to see whether possible deficiency of antigenic specificities can be related to any of these characteristics.

Materials and methods. Cell lines. The

* Supported by Grants CA-03720-09 and AI-06342 from USPHS and by Parke, Davis & Co., Detroit, Mich.

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LM strain is a subline of Strain L (929) C3H mouse cells(9). It has been maintained in monolayer in modified medium 199 supplemented with 0.5% peptone (called 199P)(4). These cells also have been adapted to grow in 2× Eagle basal medium (called 2×E)(5). The number of times that the cells have been subcultured is indicated by a prefix and the medium in which they are grown is indicated by a suffix, *e.g.*, 223 LM (2×E) cells.

The LMa cell strain had been derived by intramuscular passage of LM cells in C3H/Andervont mice(6). Clones were isolated from a culture of LMa cells plated in 199P and 20% horse serum. The clones were designated 1, 2, and 3. Clone 1 was thought to be alkaline phosphatase negative, clone 3 positive and clone 2 mixed. The morphology of the cloned cells is as follows:

Clone 1. very fibroblastic
Clone 2. fibroblastic and polygonal
Clone 3. mostly polygonal

To make an even suspension the cells were gently scraped off the glass surface with a rubber policeman, and triturated to break up cellular aggregations. The LM (2×E) cells were treated for 5 minutes in medium containing 0.12% methyl cellulose, prior to harvest, which reduces damage to the cells. LM (199P) cells were not treated with methyl cellulose as they seem to be less fragile.

Antisera. For the methods of preparation and evaluation of antibody specificities, see Gangal, Merchant and Shreffler(1). The authors would like to thank Dr. G. D. Snell, Jackson Laboratory, Bar Harbor, Maine, for the (C3H.SW × A.CA)F1 anti A.SW and (A.SW × C57BL/10)F1 anti A.CA sera.

Indirect fluorescent antibody (FAB) test. The details of this method were given to us by Dr. Nelda B. Holmgren, Dept. of Microbiology, Univ. of Michigan (personal communication). The cells in suspension were washed 3 times at 4°C in 0.1 *M* phosphate buffered saline (0.9%) at pH 7.2 (PBS) containing 0.01% $MgSO_4$. The cell concentration was then adjusted to about 2×10^6 cells per ml.

The cells were not fixed: for the test a cell viability in excess of 90% was desirable. The cells were sensitized by exposing 0.1 ml of cell suspension to 0.1 ml of dilution of antiserum at room temperature for half an hour with occasional shaking. Excess antibody was removed from the cells by washing 3 times as before(leaving them suspended in 0.1 ml PBS.

The sensitized cells were then labeled by adding 0.1 ml fluorescent horse anti mouse globulin (FHAMG) diluted 1 in 10 in PBS without $MgSO_4$. The cells were again incubated for half an hour at room temperature with occasional shaking. The FHAMG was manufactured by Progressive Laboratories Inc., and supplied by Roboz Surgical Instrument Co., Washington, D. C. The antibody was conjugated with fluorescein isothiocyanate and freeze-dried by the manufacturers.

Excess FHAMG was removed by washing again 3 times in saline as before, and the cells were mounted on slides under coverslips and sealed. The slides were then examined with a Zeiss Standard GFL fluorescent microscope equipped with an Osram HBO 200 W lamp, a BG 12 exciter filter and Zeiss barrier filter inserts (47 and 50). Some photographs were taken with a 35 mm camera using Kodak Tri X Pan fast black and white film (TX-135-36) (ASA 400). The exposure time was 40 seconds.

There are 4 types of reaction of the cell to this fluorescent staining procedure(3): (1) *Positive (Ring) reaction.* Cell takes up antibody on surface. Bright circumferential ring of fluorescence (see Figure 1). (2) *Negative reaction.* Cell takes up no antibody. No fluorescence. Cell looks dark brown all over. (3) *Diffuse reaction.* Dead cell. Fluorescence taken up non-specifically throughout cytoplasm (see Figure 1). (4) *Pinocytosis.* If cells are inactivated in FHAMG too long, pinocytosis occurs and small globules of fluorescent antibody can be seen at edge of cell. This also occurs in control tubes.

The experiment can be controlled by showing that no ring reaction appears if PBS or normal mouse serum is substituted for antiserum. This shows that FHAMG is not taken up non-specifically by LM cells.

The antisera that were used were multispecific, but each contained antibody to only one of the specificities of the $H\text{-}2^k$ allele. For

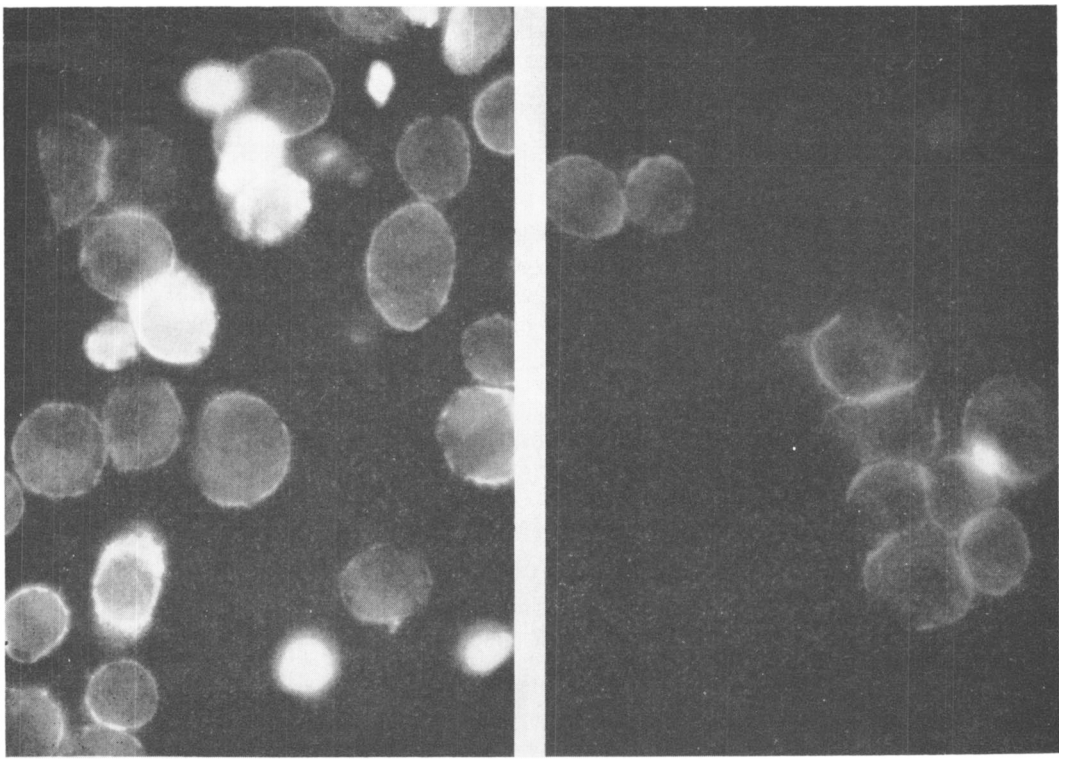

FIG. 1. Cells sensitized with undiluted antiserum and stained with 1/10 dilution of fluorescent horse anti-mouse globulin. Cells on left are 144 LM (199P) sensitized with DBA/2 anti C57BL/10 (2,5,22,33). Cells on right are 93 LMa (199P) Clone 1 sensitized with (C3H.SW × A.CA)F_1 anti A.SW (3,19).

example, DBA/2 anti C57BL/10 serum contained antibodies to specificities 2, 5, 22 and 33. It caused a positive ring reaction in indirect FAB tests, indicating that the cell had at least one of these 4 specificities on its surface. This was controlled by using C3H anti C57BL/10 serum which contained antibodies to specificities 2, 6, 14, 22, 27, 28, 29, and 33 and produced a negative reaction in FAB tests. This showed that the positive reaction caused by DBA/2 anti C57BL/10 serum was brought about by the anti 5 which it contained. Thus specificity 5 was shown to be present on the surface of the cell.

Test for alkaline phosphatase activity. This was a modification of the azo-dye method of Burstone(10). The reagents used are listed by Merchant, Kahn, and Murphy(11). The cells were not fixed. A volume of cell suspension containing about 6×10^5 cells was centrifuged in a small test tube and the medium was removed. The cells were suspended in 0.5 ml of reaction mixture and incubated at room temperature for 20 minutes. Counts were made in a haemocytometer to determine the percentage of the cells that stained blue, indicating alkaline phosphatase activity.

Results. The indirect FAB method was applied first to LM (199P) cells and they were shown still to possess specificities 1, 3, 5, 8, 11 and 32. A preliminary screening test indicated that LM (2×E) cells also had these specificities (see Table I).

A series of titrations then showed the presence of these specificities on 139-146 LM (199P) cells, 220-224 LM (199P) cells and 222-224 LM (2×E) cells. They were also demonstrated on the 3 clones of LMa cells. Titrations were not performed in the case of LMa (199P) clone 2, but the presence of the antigen was confirmed using a standard concentration of antiserum of 1/10 (see Table II).

TABLE I. Presence of H-2 Specificities on LM Cells.

Antisera*	H-2^k specificity under examination	Intensity of ring reaction 140 (LM) 199P	122 LM (2×E)
B10-D2 anti F/St (1, 7, 16)†	1	++(+)	++(+)
C3H anti P/J (6, 7, 16)		—	NT
C3H.SW-A.CA anti A.SW (3, 19)	3	+++	++(+)
A/He anti A.SW (7, 19)		—	NT
D2 anti B10 (2, 5, 22, 33)	5	++(+)	++
C3H anti B10 (2, 6, 14, 22, 27, 28, 29, 33)		—	NT
A.SW-B10 anti A.CA (8, 9)	8	+++	+++
No control serum available			
B10-D2 anti D1 (11, 17, 30)	11	+++	++(+)
C3H anti D1 (6, 13, 17, 27, 28, 29, 30)		—	NT
A/He anti C3H (32)	32	+(+)	+(+)
Monospecific serum			
Normal mouse serum	Control	—	—

* B10 = C57BL/10, D2 = DBA/2, D1 = DBA/1, B10-D2 = (C57BL/10 × DBA/2)F_1, etc. This applies to Table II and Fig. 2 also.
† The specificities towards which antibodies in this serum are directed.
NT = Not tested.

In each tube of every titration over 100 cells were examined under the fluorescent microscope to determine whether the reaction of any cells differed from that of the majority of the population. In no tube showing a strongly positive ring reaction was it possible to state with certainty that an antigen negative cell existed, and in no control tube where the reaction was negative, was a cell showing a positive reaction ever seen. Around the end-point the ring reaction was very weak, and readings fell from 100% of cells having a positive reaction to 100% of cells having a negative reaction in about 2 dilution steps (Fig. 2). Reading the dilution at which 50% of the cells are positive is a good guide to the end-point. The alkaline phosphatase activities of several cell passages were determined. The LM (2×E) cells showed no activity and the LMa (199P) clone 3 cells were 100% alkaline phosphatase positive. Activities of the cell strains are shown in Table II.

Discussion. The results of Gangal, Merchant, and Shreffler(1) have been confirmed and extended. It was shown that 139-146 LM (199P) and 220-224 LM (199P) cells have the same H-2 antigenic specificities in spite of, for some reason, widely varying alkaline phosphatase activity. The 3 clones of LMa cells also were shown to have the H-2 specificities characteristic of the *H-2^k* allele.

LM (2×E) cells were shown to have retained all the specificities, including species specificities of the LM cells. The species antigens of the LM (2×E) cells were demonstrated by showing that they could completely absorb all the antibodies to LM (199P) cells from an anti LM (199P) cell serum made in the rabbit. This was shown using a fluorescent sheep anti rabbit globulin.

Indirect FAB tests on red cells gave very weak fluorescence confirming previous results, including those of Möller(3), that red cells appear to contain relatively little transplantation antigen.

Percentage counts indicated that none of the cells are antigen negative. The great variation in alkaline phosphatase activity between different cell strains and between different cells of the same strain, contrasts with their antigenic constancy. It is apparent that alkaline phosphatase activity is not associated with gain or loss of H-2 specificities. Neither is the reduced modal number of chromosomes nor the appearance of new marker chromosomes in LM cells after transfer to 2×E medium(6) accompanied by any change in H-2 antigenic specificity.

The persistence of the *H-2^k* specificities in LM cells contrasts with the instability of the human blood group A and B specificities in some human cell lines, and with the heterogeneity of human blood group A specificity in rabbit cell lines; in the latter case heterogeneity was seen in cell lines cloned from either antigen negative or antigen positive cells(12).

No ring reaction was seen in controls what-

TABLE II. Alkaline Phosphatase Activity and H-2 Specificities of LM Cell Strains.

Cells	222-224 LM (2×E)	139-146 LM (199P)	93-94 LMa (199P) Clone 1	90 LMa (199P) Clone 2	220-224 LM (199P)	91-92 LMa (199P) Clone 3
% of cells alkaline phosphatase positive (avg)	0	13	18	71	93	100
Antisera*	Highest dilution of antiserum at which ring reaction occurs					
B10-D2 anti F/St (*1*, 7, 16)†	40	32	40	+++	32, 80	40
C3H.SW-A.CA anti A.SW (*3*, 19)	80	80, 80	80	+++	32	80
D2 anti B10 (2, *5*, 22, 33)	20	64	40	+++	32, 40	40
A.SW-B10 anti A.CA (*8*, 9)	40	80	40	+++	32, 80	40
B10-D2 anti D1 (*11*, 17, 30)	40	32	40	+++	40, 40	40
A/Hc anti C3H (*32*)	40, 16	40	10	+++	10	20

* See footnote on Table I.
† Specificity italicized is the one under examination.

ever the concentration of antiserum. Möller (3) described a few positive reactions in controls, using bone marrow and lymph node cells of adult mice but not those of 15-20-day-old mice. He related this to cellular antibody formation. It seems that LM cells produce no immunoglobulin that subsequently adheres to the cells. The cells were always washed before testing and any globulin produced by the cells may have been eluted. Möller washed his cells only once.

The suggestion that H-2 substance may be vital to the viability of the cell(1) is supported by failure to find any antigen negative cells. So far H-2 antigens have not been proved to be associated with any definite en-

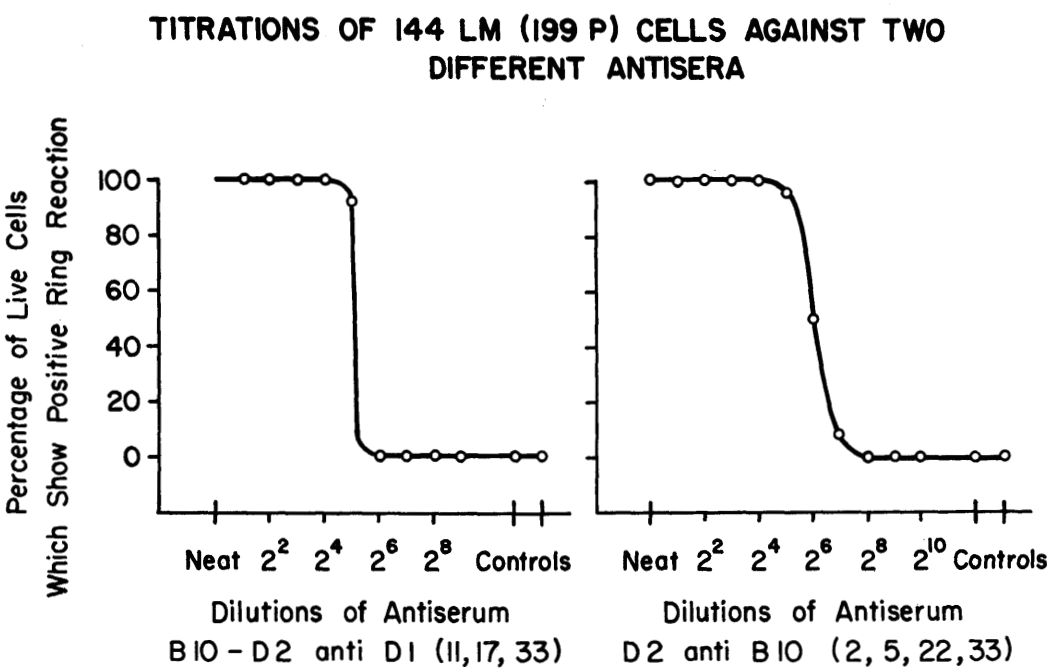

FIG. 2. For complete designations of antiserum see footnote, Table I.

zymatic activity. It may be that the H-2 antigens are part of the structural material of the cell membrane(13).

Summary. The distribution of H-2 specificities on LM mouse cells and derived lines was studied using an immunofluorescent technique. All specificities of the $H\text{-}2^k$ allele were found to be distributed uniformly among cells of all lines studied regardless of the presence or absence of alkaline phosphatase and of marker chromosomes. Variations in modal number of chromosomes and in morphology and growth characteristics of the cells also were unrelated to the presence of H-2 antigens.

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Received October 14, 1966. P.S.E.B.M., 1967, v124.

Increased Graft-*Versus*-Host Susceptibility of Thymectomized Recipients.* (31754)

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As determined by the Simonsen assay of histocompatibility(1), spleen cells derived from adult mice, thymectomized shortly after birth, fail to elicit graft-*versus*-host reactions. Previous experiments in this(2) and other laboratories(3) indicate that early thymectomy of the recipient facilitates and increases the severity of the immunological disease produced by administration of normal splenic cells.

Gross spleen enlargement after inoculation of immunologically competent cells has provided a sensitive assay system for measurement of GVH reactions by cells capable of interacting with antigens present in the host. Earlier studies(2) in our laboratory indicate that neonatally thymectomized mice are more susceptible to the lethal effects of graft-*versus*-host reactions than are normal controls. Recent work(4,5) has shown also that host cells make a major contribution to the proliferative response of spleen and other lymphoid tissues undergoing the graft-*versus*-host responses. It seems of interest, therefore, to determine whether neonatally thymectomized mice can respond with the development of splenomegaly following injection of splenic cells from adult immunologically competent donors. Experiments were designed to compare the susceptibility of thymectomized and sham operated recipients to produce splenomegaly. In this paper we attempt to demonstrate that thymectomy of the recipient ani-

* Aided by grants from Am. Cancer Soc., Nat. Foundation, USPHS (HE-02085, AI-00798, NB-02042, CA-3511), and Am. Heart Assn.

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