DEMONSTRATION OF MOUSE ISOANTIGENS AT THE CELLULAR LEVEL BY THE FLUORESCENT ANTIBODY TECHNIQUE*

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Several serological methods are available for the study of cell-bound isoantigens in mice. Hemagglutination (11) and hemolysis (14) can be used with erythrocytes while reticuloendothelial cells and certain tumor cells are responsive to the cytotoxic technique of Gorer and O'Gorman (10). Complement fixation (1) has also been employed with various kinds of cells. Most of these methods have a rather limited range of application, however. Hemagglutination and hemolysis are the most sensitive, but they can only be applied to isoantigens present on red cells, primarily the H-2 (histocompatibility-2) system. Even as regards this system, some antigens are present on tissue cells only and cannot be demonstrated on erythrocytes (26). The cytotoxic technique is restricted to the comparatively few cell types sensitive to humoral isoantibodies. The possibilities of the complement fixation method have not been explored as yet in detail.

The limitation of the previously used techniques motivated a search for sensitive methods applicable to all cell types and to a wider range of isoantigens than those represented by the H-2 system. It is desirable to demonstrate at least some of the numerous "weak" histocompatibility systems, so far mainly studied by transplantation experiments (29).

Since its original discovery, the fluorescein-conjugated antibody technique (8) has found a wide application in many fields of immunology. Several recent papers dealt with the detection of isoantigens on human cells. Successful demonstration of A, B, and a variety of Rh antigens was achieved with red blood cells (6, 16). The presence of D(Rh_o) antigen was demonstrated in human leukocytes by Jankovic and Lincoln (17) and the distribution of A, B, H, and Le^a substances in various organs was studied on histological sections by Glynn and Holborow (9). The purpose of the present work was to explore the applicability of the method for the demonstration of isoantigens in mice.

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Materials and Methods

Mice.—Cells from adult mice of the following inbred strains were used for the serological tests: A/Sn (H-2^a), C3H/Kl (H-2^k), C57Bl/Kl (H-2^b), C57L/Kl (H-2^b). In some cases the cells were taken from F_1 hybrids between two of these strains. Antisera were prepared as described below, in mice of the genotypes A.CA (H-2^f), A.BY (H-2^b), A.BY x A.SW F_1 (H-2^bH-2^s), A.BY x A F_1 (H-2^bH-2^a), C3H, DBA/2 (H-2^d), and C57L.

The breeding nuclei of all strains were maintained by strict single-line brother-sister mating. In order to maintain a high degree of homozygosis, the experimental animals were never permitted to deviate from the inbred nucleus by more than 2 brother-sister generations. All mice were vaccinated with living vaccinia virus against ectromelia at the age of 3 to 4 weeks.

The isogenic resistant lines of Snell (A.CA, A.BY, and A.SW) should be theoretically isogenic with the exception of a single difference at the H-2 locus. Transplantation studies by Linder and Klein (21) showed, however, that the theoretical expectations were not fulfilled altogether in practice, since skin transplants were regularly rejected in intrastrain graftings, indicating that the strains were not homozygous with regard to several weaker histocompatibility loci. However, intrastrain tumor grafts grew progressively in nearly 100 per cent.

Tumors.—

TA3: This tumor has originated as a spontaneous mammary adenocarcinoma in a female A mouse in 1949. It was carried by subcutaneous transfers in A mice during the first 16 generations and showed a gradual loss of differentiation. After the 16th generation it was converted to the ascites form and subsequently carried intraperitoneally in A mice. It kills 100 per cent of strain A mice, but does not kill mice of foreign strains. During the present experiments the tumor was between its 190th and 202nd transfer generation. Solid tumors were produced when needed by intramuscular grafts.

MC1M: This tumor was induced by methylcholanthrene in a C3H mouse in 1946. It started out originally as a rhabdomyosarcoma and was transformed into the ascites form in 1951 (20). Since then it has been maintained by intraperitoneal passages in strain C3H mice for 215 generations. It kills 100 per cent of C3H mice and grows to a limited extent also in foreign strains.

6C3HED: The lymphosarcoma 6C3HED originated several decades ago in a female C3H mouse treated with estradiol benzoate at Gardner's laboratory. It has been transformed into the ascites form (20) in 1951 and was carried since then by intraperitoneal injections of ascites tumor cells in strain C3H. It takes in 100 per cent of C3H mice which survive for 9 to 11 days.

5L1: This lymphoma originated in 1959 in a 1-year-old C57Bl male which had been x-irradiated with 100 r at birth and with 300 r 3 months later. It has been carried by serial subcutaneous passage in C57Bl mice for 27 generations. It grows progressively in and kills 100 per cent of C57Bl mice. Material for tissue sections was taken from intramuscular transplants as a rule.

MALA: The solid sarcoma MALA was induced in 1959 by methylcholanthrene in the thigh of an A x C57L F_1 female mouse. It has been carried by subcutaneous passage in A x C57L F_1 mice for 22 generations. For fluorescent antibody studies on tissue sections material was taken from intramuscular grafts as a rule.

MC57C: This solid sarcoma was induced in 1959 in a C57Bl female by methylcholanthrene. It has been maintained by subcutaneous transplantations in C57Bl mice for 18 passages.

Preparation of Tissue Sections.—Sections were made from subcutaneous or intramuscular grafts of tumors TA3, 5L1, and MALA. The tumors were removed and blocks were cut with an approximate area of 0.5 cm^2 and 3 to 5 mm thickness and were immediately frozen with

carbon dioxide snow on a microtome object holder. The frozen tissue blocks were cut at an indicated thickness of 4 μ in a cryostat at −20°C. The sections were placed on chilled slides and rapidly thawed at room temperature by placing a finger under the slides. Subsequently the slides were immersed either in Ringer's solution or in 96 per cent ethanol for 10 minutes. The latter procedure was only employed when the sections were designated for counter staining with rhodamine-conjugated bovine albumin.

Preparation of Cells.—Ascites tumor cells were washed once in Ringer's solution containing 100 IU of penicillin and 100 μg streptomycin/ml and then diluted to a final concentration between 5 and 20 X 10^6 cells per ml as determined by cell counts in a hemocytometer. Cell suspensions from solid tumors, lymph nodes, and spleen were prepared by pressing the tissues through a 60 mesh stainless steel screen into Ringer's solution; larger particles were allowed to sediment and the remaining free cell suspension was washed once and then diluted to a final concentration varying from 1 to 20 X 10^6 cells per ml.

Cell suspensions from ear epidermis were prepared by exposure to 0.25 per cent trypsin solution for 1 hour at 37°C as described by Billingham and Reynolds (4).

Red blood cells were taken from the retro-orbital sinus through a glass capillary and suspended in 4 per cent sodium citrate solution. They were washed once in Ringer's solution and then resuspended to a 2 per cent *v/v* concentration in Ringer's solution.

Preparation of Antisera.—H-2 isoantisera were produced in strains A.CA, C3H, DBA/2, and C57L by repeatedly inoculating them with cell suspensions derived from spontaneous mammary carcinomas, spleen, lymph nodes, and liver from foreign H-2 genotypes. Three to ten inoculations were given intraperitoneally and subcutaneously at intervals of 6 to 10 days. 6 to 7 days after the last inoculation blood was collected from the retro-orbital sinus through a glass capillary. Volumes between 0.4 and 0.8 ml were taken from each animal. Blood pooled from 4 to 14 mice was allowed to clot at room temperature. The pooled sera were kept at −20°C. The following isoantisera were used in the tests: A.CA anti-C3H (hemagglutination titer: 1/1024), C57L anti-A (1/1024), DBA/2 anti-C57B1 (1/512), and C3H anti-A (1/512).

Isoantisera against other antigenic systems than H-2 were produced in the genotypes A.BY (H-2^b), A.BY x A.SW F_1 (H-2^bH-2^s), A.BY x A F_1 (H-2^bH-2^a), and C57L (H-2^b) by immunizing them against two strain-specific C57Bl tumors: 5L1 and MC57C. The antigenic systems involved are numerous but they are all non-H-2.

Immunization against 5L1 and MC57C was performed by repeated subcutaneous inoculations of cell suspensions at intervals of 6 to 35 days. Twenty challenges were given with 5L1 and eighteen with MC57C. 7 to 20 days after the last inoculation blood was collected from the retro-orbital sinus. When tested by the cytotoxic method of Gorer and O'Gorman the sera gave a weakly positive cytotoxic index (between 0.13 and 0.26) defined as the difference between the proportion of unstained cells in the control and the test suspension, divided by the former figure (13). No positive hemagglutination reactions were obtained with these sera (13), using the test of Gorer and Mikulska.

Fluorescein Conjugates.—A commercial preparation of fluorescein-conjugated rabbit anti-mouse globulin (Microbiological Associates, Bethesda lot 13.570) was used.

The conjugated serum was either undiluted or diluted 50 per cent in Ringer's solution. When tissue sections were studied the serum was absorbed twice for half an hour at room temperature with the same volume of acetone powder of mouse liver prepared according to the method of Mellors (23). Initially, the same absorption procedure was also carried out when studying living cells, but this was later abandoned since it was found, as described below, that non-specific staining was either absent or, if present, it was of a different nature and could not be affected by the absorption to any detectable degree.

Staining Techniques.—The indirect or "sandwich" method was used throughout. Unfixed, thawed tissue sections were covered with 0.05 to 0.1 ml of the appropriate isoantiserum. They were incubated for 15 to 30 minutes in a moist chamber at room temperature, washed three

times with an excess of Ringer's solution, and then covered with the same amount of the conjugated rabbit anti-mouse serum for the same time. After three more washings the sections were mounted in a medium consisting of 9 parts of anhydrous glycerin and 1 part of Ringer's solution, and immediately inspected in the fluorescent microscope.

In some cases the sections were prefixed with ethanol as described above. Fixed sections were exposed to mouse isoantiserum in the same way but subsequently they were treated with a mixture of 5 parts rabbit anti-mouse serum conjugated with fluorescein isothiocyanate and absorbed with mouse liver powder, and 1 part unabsorbed rhodamine-conjugated bovine albumin (Microbiological Associates, lot 16.746).

Two kinds of living cell preparations were used. In one the cells were brought into suspension as described above, diluted to a concentration of 10^6 per ml in Ringer's solution, and allowed to settle down on a coverglass for 1 hour at 37°C in a moist chamber. The sedimented cells were washed in Ringer's solution and covered with isoantiserum. Not all cell types adhere to the coverslip firmly enough, however, and some tend to be washed away during the subsequent procedure. Best results were obtained with bone marrow cells, while lymph node cells were somewhat less satisfactory and ascites tumor cells even less suitable.

The second procedure involved the treatment of suspended living cells. This technique worked well with all cell types tested unless the frequency of dead cells was too high (over 50 per cent). 1 ml aliquots of cell suspension containing 5 to 20 X 10^6 cells per ml were centrifuged at 150 *g* for 2 minutes in small test tubes. The supernatant was replaced with isoantiserum in dilutions varying between 1/2 and 1/6 in different experiments. The sediment was brought into suspension by shaking and the suspension was incubated at room temperature for about 15 minutes. After being washed twice with 2 ml lots of Ringer's solution, the cells were incubated with 0.05 to 0.1 ml conjugated anti-mouse globulin for another 15 minutes at room temperature. During the incubation the tubes were agitated at intervals of 2 to 4 minutes. After two more washings the material was diluted to the original concentration in Ringer's solution. It was kept in an ice bath until inspected in the fluorescent microscope within half an hour. The coverslip preparations were handled in an analogous manner. Microscopic examination was carried out on living cells, lying between a coverslip and a slide. About 0.05 ml of the cell suspension was placed on the slide and overlaid with a coverslip. Care was taken to avoid pressure of the coverslip against the slide. No injuries of the cells due to pressure were observed. Since many cells and ascites tumor cells in particular tended to swell during inspection in ultraviolet light, some preparations were fixed by exposing the cell suspension to 95 per cent ethanol for about 10 minutes at room temperature after the staining procedure was completed. This procedure preserved the cells well and did not change their fluorescence or other morphological details visible in the fluorescent microscope. The swelling of the unfixed cells did not interfere with the interpretation of the staining reaction but it hampered microphotography.

The staining procedure for living cells was worked out with the specific purpose to minimize non-specific reactions including those due to pinocytosis. The latter was studied by incubating cell suspensions at 37°C with fluoresceinisothiocyanate–conjugated normal mouse serum for periods varying from 15 minutes to 5 hours. All cell types used were compared with regard to their pinocytosing ability. Cell concentrations and all other experimental conditions with the exception of the incubation period were the same as in the immunological studies. The conjugated normal mouse serum was prepared according to the procedure described by Riggs *et al.* (28). It was absorbed twice with acetone powder of mouse liver before use. After incubation the cells were washed twice and then inspected in the fluorescent microscope.

Microscopy.—A Zeiss fluorescent microscope was used. The source of ultraviolet light was as Osram HBO 200 high pressure mercury lamp. Two BG 12 exciter filters and OG 4 and OG

5 barrier filters were used. All specimens were examined alternatively in ordinary light and in ultraviolet light with two objectives (apertures 0.32 and 0.63) and a 10 X ocular. Black and white photographs were taken with Kodak plus-X panchromatic film and color photographs with Kodak high speed ectachrome daylight film. Exposure times varied between 3 and 6 minutes for both films.

Controls.—The following controls were examined in order to distinguish between specific reactions and non-specific staining.

1. The isoantiserum chosen to react with a certain cell type of the foreign genotype against which it was directed was also incubated, under identical conditions, with either the same cell type from the antiserum donor strain, or, if tumor cells were the target of the specific reaction, with normal bone marrow or lymph node cells. In the experiments with sectioned solid tumors another tumor derived from the antiserum donor strain or another strain with the same H-2 genotype was used as a control.

2. The isoantiserum was replaced with normal mouse serum or with an antiserum that was not specifically directed against the isoantigens of the cells examined but was prepared by immunizing mice of the cell donor strain with cells of another foreign strain. This control was included in most experiments with tumor cells and tissue sections.

3. The isoantiserum was replaced with Ringer's solution. This was followed by the exposure to the fluorescein-labeled rabbit anti-mouse serum. This control was included in all experiments.

At least two of these controls were included in every experiment.

RESULTS

A. Staining Reactions

Injury Reaction.—In all experiments with living cells there were some cells showing diffuse fluorescence (Figs. 5 to 8). This reaction was described by Holtzer and Holtzer (15) and was shown to be due to cell injury and subsequent staining of the damaged cells by the fluorescent protein solution. The readiness with which injured cells bind fluorescein-labeled rabbit anti-mouse serum was confirmed in experiments where MC1M ascites tumor cells were killed by incubation at 56°C for 2 hours and subsequently incubated with the fluorescein-conjugated rabbit anti-mouse serum globulin for 15 minutes at room temperature, washed three times, and then inspected in the fluorescent microscope. About 90 per cent of the cells were diffusely stained. In the control MC1M suspension which was treated in an analogous manner except for the incubation at 56°C only about 2 per cent of the cells showed diffuse staining, the rest remaining completely unstained. The injured cells showed the same staining reaction when treated with fluorescein-conjugated normal mouse serum and this must therefore be regarded as a non-immunological reaction. It is probably analogous to the staining reactions with eosin, trypan blue, nigrosine, lissamine green, etc., routinely used to demonstrate dead cells in cell suspensions by virtue of the altered permeability of the cell membrane.

The cytological detail of the injury reaction showed little variation with the various kinds of cells used. In general, staining was diffuse and no morphological details could be seen (Figs. 5 to 7). In some cases the nuclei were stained

more intensely than the cytoplasm and sometimes the fluorescent material was concentrated into one or several large clumps, the remaining parts being more weakly stained. In most cases this type of staining was weaker in intensity than the specific and morphologically different "ring" reaction which will be described below. Furthermore the intensity of the injury reaction diminished even more when the fluorescent serum was absorbed with acetone powder of mouse liver. The latter procedure has not been performed routinely, however, because the non-specific staining of the injured cells did not interfere with the specific "ring" reaction.

Pinocytosis.—In order to study the possible contribution of pinocytosis to the staining reaction, cell suspensions from different tumors and from lymph nodes and bone marrow were incubated with fluorescein-conjugated normal mouse serum for various times. With the exception of the incubation period all other conditions were identical with the immunological experiments.

The morphological signs of pinocytosis were essentially the same with all cells studied. The cytoplasm became more or less filled with small fluorescent vacuoles, ranging in size from about 1 to 3 μ, which did not coalesce (Fig. 8). As a rule, the vacuoles tended to accumulate in the juxtanuclear area. The nuclei did not show fluorescent staining in any of the cells studied.

The proportion of cells showing the pinocytosis reaction was different for different cell types. Lymph node and bone marrow cells displayed the most extensive uptake of this type (28 to 51 per cent after 2 hours' incubation). The frequency of positive cells varied in different experiments. The tumor cells studied showed the pinocytotic uptake to a limited extent only (0 to 2 per cent with MC1M, 0 to 6 per cent with TA3, 1 to 4 per cent with 6C3HED, and 4 to 6 per cent with 5L1). There were slight morphological differences between the appearance of the fluorescent vacuoles in normal lymph node and bone marrow cells on the one hand and tumor cells on the other. As a rule, the normal cells contained smaller vacuoles and these were often present in larger numbers.

The first cells showing the pinocytosis reaction were seen after 30 minutes' incubation in the lymph node and bone marrow suspensions. They increased in frequency upon continuing incubation and the previously mentioned levels were reached after 2 hours. In tumor cell suspensions the reaction was rarely seen earlier than 1 hour.

In order to assess whether isoantibodies directed against H-2 antigens may exert any influence upon pinocytosis, MC1M and TA3 ascites tumor cells were incubated for periods varying from 30 minutes to 2 hours with A.CA anti-C3H and C57L anti-A isoantiserum, respectively, together with fluorescein-conjugated normal mouse serum. The proportion of cells showing pinocytosis did not change. Because of its entirely different morphological appearance, there was no difficulty in distinguishing the pinocytosis reaction from the specific immunological staining that will be described below, or from the injury reaction discussed above.

Specific Immunological Reaction: "Ring" Staining.—

(*a*) *Living cells:* In order to facilitate the demonstration of specific immuno-

logical reactions, care was taken to avoid the non-specific staining due to pinocytosis by restricting the maximum incubation time to 15 minutes.

Living cells were studied both in suspension and in preparation where they adhered to coverslips. The specific staining reaction was identical in appearance in both preparations. Cells pretreated with isoantiserum and subsequently exposed to the fluorescent rabbit anti-mouse serum showed a bright green-yellow fluorescence outlining clearly the cell surface (Figs. 3, 4, 6, 9, 10, and 12). The "ring" reaction contrasted sharply with the diffuse staining of the injured cells (Fig. 6) which were present in varying numbers in all preparations, but did in no way disturb the identification of the specific reaction. The injured cells usually exhibited a much weaker staining and they were present in the controls and the antiserum-treated preparations to the same extent. In no case was there any indication of any cytotoxic effect in the antiserum-treated cell suspensions. This can be attributed to the fact that mouse serum is devoid of complement activity *in vitro* (26).

Cells with a positive "ring" reaction were quite uniform in appearance and the different cell types studied could not be distinguished by morphologically different reaction types in contrast to the non-specific reaction described in the previous sections where the detailed picture often varied from tissue to tissue. The intensity of the "ring" reaction varied little from cell to cell in tumor cell populations; in lymph node and bone marrow preparations, however, there was often considerable variation between different cells with regard to the intensity of the fluorescence.

The "ring" reaction was completely absent from all controls, with the exception of bone marrow and lymph node suspensions, where a small minority of the cells showed a typical "ring" reaction without having been exposed to specific isoantisera (Fig. 11). All three types of controls behaved similarly in this respect. The "ring" reaction was often incomplete in these preparations, however, with only part of the cell membrane stained, giving the appearance of fluorescent crescents. Such crescents were never observed in lymph node and bone marrow suspensions pretreated with specific isoantiserum where nearly all cells regularly showed the "full" ring reaction which was also characterized by a brighter fluorescence. The non-specific reaction in the controls could be made even less intense by repeated washings of the cells in Ringer's solution prior to incubation with the fluorescent rabbit anti-mouse serum, although it was never completely abolished. It was not affected by absorbing the fluorescent rabbit serum with acetone powder of mouse liver. The total proportion of control cells showing the complete or incomplete "ring" reaction varied between 5 and 15 per cent in different experiments.

(*b*) *Tissue sections:* In sections treated with specific anti-H-2 isoantisera the intercellular boundaries were clearly outlined by greenish-yellow fluorescence (Fig. 1). Nuclei always failed to stain. It was not possible to decide whether

the staining was localized at the cell membrane and/or in the cytoplasm. The staining was always much less bright than with living cells. The controls showed only a very faint diffuse background staining. When ethanol-fixed sections were stained with a mixture of fluorescein isothiocyanate–conjugated rabbit anti-mouse serum and rhodamine-conjugated bovine albumin, the controls (Fig. 2) displayed a homogeneous red staining while the sections pretreated with the specific antiserum showed green fluorescence of the intercellular boundaries, often with faintly reddish nuclei (Fig. 1).

B. Demonstration of Isoantigens on Different Cells

Erythrocytes.—Red blood cells of A and C3H mice were tested against C57L anti-A and A.CA anti-C3H serum, respectively. The staining procedure was carried out with 0.05 to 0.1 ml of a 0.5 to 2.0 per cent cell suspension. The high dilution was necessitated by the finding that red cells stained very weakly and the specific reaction was obscured if too many cells were used. Cells from the antiserum-donor genotype were used as controls. Antiserum-treated cells showed a weak but clear green-yellow fluorescence of the "ring" type (Fig. 9). No diffusely stained cells were seen. Stained cells could not be detected in the controls and non-specific staining reactions appeared to be entirely absent. The comparatively weak specific staining reaction may be tentatively interpreted to indicate a possible difference in the quantity of isoantigens at the cell membrane of red cells. This view is supported by the finding (25) that erythrocytes have a low capacity of absorbing isoantibodies compared to spleen, liver, and kidney cells.

After having been treated with an H-2 isoantiserum, the erythrocytes tended to agglutinate when exposed to the fluorescent rabbit anti-mouse serum. A weaker agglutination was sometimes present in the controls also. These control agglutinates did not show any fluorescence; this argues against the possibility that the fluorescence of the antiserum-treated agglutinates was due to a non-specific trapping phenomenon. A similar conclusion was reached by Cohen *et al.* (7). These authors were interested in the identification of minor cell populations by blood group antigens and used the fluorescent antibody method. They concluded that trapping is not likely to be a source of confusion in the interpretation of pictures obtained by exposing red cells to labeled antibodies nor does it intensify the fluorescence obtained in the presence of specific antibody.

Epithelial Cells.—Ear epidermis cells of A × C3H F_1 hybrids and C3H mice were brought into suspension by trypsin treatment. 10,000,000 cells were exposed to A.CA anti-C3H serum. Normal mouse serum replaced the antiserum in the controls as far as the A × C3H F_1 cells were concerned, while C3H anti-A serum was used as control with the C3H cells. Antiserum-treated cells showed a strong "ring" reaction of rather uniform intensity (Fig. 10). The proportion of cells showing the injury reaction varied in the two experiments (10 and 50 per cent, respectively), with no significant difference between the

corresponding controls and antiserum-treated suspensions. No pinocytotic reaction could be observed. No "ring" reaction was seen in the controls.

Lymph Node and Bone Marrow Cells.—Suspended cells and coverslip preparations were examined as well. A, C3H, and C57Bl cells were exposed to C57L anti-A, A.CA anti-C3H, and DBA/2 anti-C57Bl, respectively. Both kinds of preparation gave similar results although the number of injured cells was often somewhat smaller in the coverslip preparations. Non-injured antiserum-treated cells showed a typical "ring" reaction in 90 to 100% (Fig. 3). Injured

TABLE I

The Occurrence of Non-Specific "Ring" Reactions with Hematopoietic Cells of Newborn and Embryonic Mice, Subsequent to Treatment with Fluorescent Rabbit Anti-Mouse Serum

Age of donor mice (strain A)	Total No. of non-injured cells counted	No. of cells showing complete and incomplete "ring" reactions	"Ring" reactions
days			*per cent*
Embryos			
15	>500	0	0
20	>500	0	0
Newborn			
1	1800	3	0.17
3	390	6	1.5
5	250	10	4.0
7	340	19	5.6
14	360	25	6.9

cells comprised between 10 and 30 per cent of the population and were diffusely stained. The "ring" reaction often varied in intensity, with a comparatively weakly stained cell minority. It cannot be decided on the basis of available evidence whether this was a technical artefact or reflected a true quantitative variation of cellular antigen concentration.

The control preparations were composed of cells from the antiserum donor genotype, incubated with the same antiserum or with normal mouse serum. All controls contained between 10 and 30 per cent injured, diffusely stained cells. In addition, 5 to 15 per cent of the cells exhibited a complete or incomplete "ring" reaction, usually less bright than in the preparations treated with specific antiserum (Fig. 11), while the rest remained entirely unstained. Apart from the difference in intensity, and its occasional incompleteness in the controls, the "ring" reaction was of the same appearance in the controls and in the antiserum-treated preparations. This suggested that the "ring" reaction of the controls was due to an immunological reaction between protein molecules bound to the cell membrane and the fluorescein-conjugated rabbit anti-mouse serum.

It was observed that hematopoietic cells obtained from the liver of 15- to

20-days-old strain A mice, presumably incapable of antibody synthesis, completely lacked the "ring" reaction when incubated with fluorescent rabbit anti-mouse serum. With spleen cells of newborn A mice the proportion of stained cells showing a non-specific ring reaction increased as a function of time after birth (Table I). It has been shown (6) that maternal antibodies are transferred to the fetus through the placenta and by nursing. The lack of non-specific ring reaction in embryonic mice and their scarcity in newborns in spite of the presumed presence of maternal antibodies decreases the likelihood of the presence of "cytophilic" antibodies attached to the cell membrane. The increasing frequency of "ring" reactions in control suspensions of newborn cells is in accordance with the assumption that the reaction is related to cellular antibody formation, since it has been shown (2) that 1- to 2-days-old A mice could respond with specific immunological tolerance when inoculated with adult cells of a foreign genotype, and were thus probably incapable of antibody synthesis. The tolerance-inducible period of newborn A mice towards H-2 isoantigens terminated after approximately 3 days; at 7 days of age evidence of weak immunity was obtained. By this time the incidence of non-specific ring reactions was 5.6 per cent in spleen cell suspensions of A mice. This is within the range (5 to 15 per cent) obtained with adult lymph node cells.

Tumor Cells.—Tumor cells were studied both as living cell suspensions and in tissue sections.

(*a*) *Living cells:* TA3 ascites carcinoma cells of strain A origin were treated with C57L anti-A serum in suspension. Subsequent to treatment with fluorescent rabbit anti-mouse serum, all non-injured cells showed a vivid green-yellow fluorescence, outlining clearly the cell surface. The "ring" reaction contrasted sharply with the diffuse and weak staining of injured cells, comprising 1 to 5 per cent of the cell population. No reaction of the pinocytosis type was observed.

Control suspensions treated with normal mouse serum were characterized by the complete absence of the "ring" reaction. The diffuse staining of the injured cells was present to the same extent as the the antiserum-treated suspensions (Fig. 5).

MC1M ascites tumor cells were tested with an A.CA anti-C3H serum. The specific "ring" reaction (Figs. 4 and 6) was of the same type as with TA3 cells. The controls were treated with normal mouse serum and never showed this reaction. The diffuse staining of injured cells (Figs. 6 and 7) was present in 1 to 5 per cent in both controls and antibody-treated suspensions.

6C3HED ascites tumor cells were treated in suspension with the same A.CA anti-C3H serum. About 95 per cent of the cells showed the specific "ring" reaction. The number of diffusely stained cells varied between 1 and 5 per cent in different experiments. Very few cells (1 to 2 per cent) were unstained; these were only visible in the light microscope. Since the intensity of the "ring" reaction varied between different cells, the lack of staining of this minority may be due to an unequal absorption of antibody by the cells. The possibility that the unstained cells are antigenic variants exists but has not been investigated.

Control suspensions treated with normal mouse serum and C3H anti-A serum showed no trace of the "ring" reaction. The number of injured cells corresponded to the antiserum-treated suspensions. Pinocytosis reactions were not observed. Control lymph node cells of strain A.CA treated with A.CA anti-C3H serum showed the usual proportion of weak "ring" reactions with this type of material (5 to 15 per cent) and were regarded as negative.

5L1 lymphoma cells were brought into suspension and treated with DBA/2 anti-C57Bl serum. With the exception of 10 to 30 per cent of the cell population, which showed the diffuse injury reaction all cell membranes were intensely stained. The same proportion of injured cells was found in the normal mouse serum controls. Pinocytosis and "ring" type reactions were absent from the controls.

5L1 lymphoma cells were also tested with isoantisera directed against isoantigenic systems other than H-2. A.BY, A.BY x A F_1, and C57L mice, all containing H-2^b, were immunized against 5L1 lymphoma cells, which, it will be recalled, have been derived from a C57Bl (H-2^b) mouse. A.BY x A.SW F_1 mice, also containing H-2^b, were immunized against sarcoma MC57C, another C57Bl tumor. Since both donors and recipients contained H-2^b in all these combinations, the antibodies produced must be directed against antigenic systems other than H-2.

All antisera were tested against 5L1 lymphoma cells and normal C57Bl lymph node cells. In two experiments, A.BY and C57L anti-5L1 sera were also tested against 5L1 after absorption with normal C57Bl lymph node cells. Normal mouse serum replaced the antiserum in one type of controls and lymph node cells of the antiserum donor strain was used instead of C57Bl cells in another. The results of these experiments are summarized in Table II. Normal C57Bl lymph node and bone marrow cells pretreated with A.BY and C57L anti-5L1 sera exhibited a typical "ring" reaction in 50 to 75 per cent. The suspensions of 5L1 lymphoma cells, tested with the same two sera and also with A.BY x A F_1 anti-5L1 serum, displayed a typical "ring" reaction in all non-injured cells. Absorption of A.BY anti-5L1 and C57L anti-5L1 sera with normal C57Bl lymph node cells led to a decrease in the frequency of specifically membrane-stained 5L1 cells from 100 to 3 and 0 per cent, respectively. The few remaining positive cells in the former case exhibited a very weak "ring" reaction. When A.BY x A.SW F_1 anti-MC57C serum was tested against 5L1 lymphoma cells, 60 per cent of the non-injured 5L1 cells showed a typical "ring" reaction.

Both MC57C and 5L1 induced the formation of antisera capable of reacting with 5L1 cells. The anti-5L1 serum was also tested against normal C57Bl lymph node and bone marrow cells and was found reactive. Since the activity against 5L1 could be removed by absorbing with normal C57Bl lymph node cells, it has to be concluded that it was not directed against tumor-specific antigens but against isoantigens present in both normal and neoplastic cells and belonging to isoantigenic systems other than H-2. The possibility that the isoantibodies studied might nevertheless be directed against some component of the H-2 system, because of differences in the H-2^b allele carried by the different strains, is unlikely, particularly since it was found that both A.BY and C57L were capable of producing antibodies reacting with C57Bl cells.

(*b*) *Tissue sections:* Sections of solid TA3 carcinoma of strain A origin and MALA sarcoma of A x C57L F_1 origin were exposed to C57L anti-A serum. Solid 5L1 lym-

TABLE II

Staining Reactions of C57Bl Bone Marrow and Lymph Node Cells and of the 5L1 Lymphoma After Pretreatment with Non-H-2 Isoantisera

Exp. No.	Cell type	Antiserum	Absorption of antiserum	"Ring" reaction shown by non-injured cells in antiserum-treated suspensions	"Ring" reaction shown by non-injured cells in controls with normal mouse serum	Remarks
1	C57Bl bone marrow	A.BY anti-5L1	None	About 75 per cent	About 10 per cent	Variable intensity of "ring" reaction
2	C57Bl lymph node cells	C57L anti-5L1	None	About half of the cell population	About 5 per cent	"
3	5L1 lymphoma cells	A.BY x A F_1 anti-5L1	None	All cells	None	Uniform intensity of "ring" reaction
	5L1 lymphoma cells	A.BY anti-5L1	None	All cells	None	"
4	C57Bl lymph node cells	"	None	Half of the cell population	About 5 per cent	
	5L1 lymphoma cells	"	Absorbed with normal C57Bl lymph node cells	About 3 per cent	None	Very weak "ring" reaction
5	5L1 lymphoma cells	C57L anti-5L1	None	All cells	None	
	5L1 lymphoma cells	"	Absorbed with normal C57Bl lymph node cells	No cells	None	
6	5L1 lymphoma cells	A.BY x A.SW anti-MC57C	None	About 60 per cent	None	Weak "ring" reaction

phoma sections (of C57Bl origin) were exposed to DBA/2 anti-C57Bl serum. Controls were treated with normal mouse serum or with antiserum produced by immunizing the strain of tumor origin with tissues of a foreign strain. In some experiments with TA3, sections of 5L1 treated with the same C57L anti-A serum were used as controls. The results were similar with all tumors used. Bright greenish-yellow fluorescence outlined the intercellular boundaries in all sections treated with specific antiserum. The general staining pattern was similar to that seen in antiserum-treated suspensions of living cells, especially when compared to agglutinates, although the intercellular boundaries were less distinctly outlined in the sections and stained considerably weaker. No nuclear fluorescence was observed in the sections. It was not possible to decide whether the fluorescence was localized at the cell membrane, or the cytoplasm, or both. Unfixed control sections treated with fluorescein-conjugated rabbit anti-mouse serum showed very faint diffuse background staining without any specific localization. When 5L1 sections were used as controls and treated with C57L anti-A serum, the same weak and diffuse background staining was obtained, indicating that non–H-2 antibodies capable of reacting C57Bl cells were either not present in this serum, or, if present, were unable to give a detectable specific staining. When ethanol-fixed sections were stained with a mixture of fluorescein isothiocyanate–conjugated rabbit anti-mouse serum and rhodamine-conjugated bovine albumin, the controls displayed a diffuse red staining (Fig. 2), while the material exposed to the specific isoantiserum showed green coloration of the intercellular boundaries (Fig. 1), often with faintly red nuclei.

There were some minor differences in staining between the three tumors studied. As a rule, TA3 and 5L1 stained more intensely than MALA. This probably reflects a difference in the surface antigen concentration of MALA and TA3, which were stained with the same C57L anti-A serum. This is in accordance with the findings that sarcomas often contain a lower concentration of H-2 surface antigens per cell than carcinomas such as TA3 (24). In addition, MALA is a heterozygous tumor and therefore probably in possession of less antigens per cell than a homozygous tumor such as TA3.

DISCUSSION

These experiments have established beyond doubt that mouse isoantigens can be demonstrated at the cellular level by the fluorescent antibody technique in various kinds of normal and malignant cells. A schematic summary of the results is presented in Table III.

When applied to histological sections, the fluorescent antibody technique is often complicated by undesirable non-specific reactions. These can be largely avoided in work with living cells, since the cell membrane tends to keep out macromolecules in short term experiments. Injured cells with impaired membrane function are, on the other hand, readily permeable and show diffuse fluorescent staining. This makes the method rather unsuitable for cell suspensions that contain a high proportion of injured cells. The appearance of injured cells is so different from the specific immunological reaction that takes place

TABLE III

Staining Reactions of Different Cell Types with the Fluorescent Antibody Technique

Cell type	Preparation	"Ring" reaction in antiserum-treated preparations	"Ring" reaction in controls	"Cell injury" reaction	Pinocytosis	Isoantigenic system	Remarks
Red blood cells	suspension	+	−	−	−	H-2	Weak "ring" reaction
Lymph node and bone marrow cells	"	+	+ (5 to 15 per cent)	+ (10 to 30 per cent)	−	H-2 and non H-2	Variable intensity of "ring" reaction in antiserum-treated preparations. 25 to 50 per cent unstained cells in non H-2 systems
Epithelial cells	"	+	−	+ (10 to 50 per cent)	−	H-2	All non-injured cells stained with "ring" reaction in antiserum-treated suspensions
Tumor cells:							
MC1M	"	+	−	+ (1 to 5 per cent)	−	H-2	"
TA3	"	+	−	+ (1 to 5 per cent)	−	H-2	"
6C3HED	"	+	−	+ (2 to 5 per cent)	−	H-2	Slight variation in intensity of "ring" reaction. 1 to 2 per cent of the cell population unstained
5L1	"	+	−	+ (5 to 20 per cent)	−	H-2 and non–H-2	Weaker staining in non H-2 systems
TA3	Tissue section	+	−			H-2	Very weak reaction in antiserum-treated preparations. Diffuse background staining in controls
5L1	"	+	−			H-2	"
MALA	"	+	−			H-2	"

at the cell membrane, however, that the two types of staining can be easily differentiated.

Upon prolonged incubation, certain cell types tend to engulf proteins from the surrounding medium by pinocytosis. This may reach considerable proportions in the course of time particularly with bone marrow and lymph node cells. Since the immunological reaction between the isoantigens studied in the present work and the corresponding isoantibodies seems to take place rather rapidly (after an incubation of 5 minutes a weak but clear positive reaction has been obtained with all cells studied) there is no need to extend the incubation period so as to permit pinocytosis (at least 30 minutes were necessary with the cell types investigated, and the morphologically distinct pinocytosis reaction was completely absent after 15 minutes with all cells).

Lymph node and bone marrow cells were unique among the different tissues studied in showing a low frequency of non-specific reactions which were often morphologically indistinguishable from the specific "ring" reaction. The great resistance of this reaction to repeated washings in Ringer's solution prior to treatment with the fluorescent rabbit anti-mouse serum indicates a strong attachment of the responsible substance(s) to the cell surface, and argues against the possibility that the reaction was due to insufficient dispersion and washing of the cells. Since hematopoietic cells from the livers of 15- to 20-days-old embryos, not yet capable of antibody formation, did not show the non-specific "ring" reaction, it appears to be related to cellular antibody-forming capacity. This is further supported by findings that with spleen cells of newborn A mice the proportion of stained cells showing a non-specific "ring" reaction increased as a function of time after birth. The most probable interpretation is an immunological reaction between the fluorescent rabbit anti-mouse serum and mouse serum proteins, possibly gamma globulins, present at the cell membrane. If so, the reaction would reveal cells engaged in antibody synthesis. Alternatively, "cytophilic" antibodies attached to the cell surface might be responsible (see reference 5). However, the lack of non-specific "ring" reactions in embryonic mice in the presumed presence of maternal antibodies decreases the likelihood of the latter explanation. The significance, if any, of the incomplete crescent-like staining reaction of some of the cells is unknown.

The sensitivity of the fluorescent antibody method in comparison with other available techniques has not been studied systematically. As far as non–H-2 differences are concerned, it seems to be more sensitive than other serological methods such as hemagglutination and cytotoxic tests, probably because its identification is not dependent on secondary reactions such as agglutination, cytolysis, or complement fixation.

Both with living cells and tissue sections, the fluorescent antibody method indicated that the isoantigens of the H-2 system are present at the cell membrane. No specific nuclear staining could be demonstrated with sectioned

material. Because of the rather diffuse localization of the staining in the sectioned material, it could not be decided whether the membrane localization of the antigens was exclusive or extended to the cytoplasm.

While most serological methods can only detect isoantigens at the population level, the fluorescent antibody method works at the cellular level. It follows that a minority of antigenic variants should be detectable in large populations lacking the antigen in question, provided that non-specific reactivity can be excluded. No such experiments have been undertaken in this study, but according to Cohen *et al.* (7) as few as 10^{-5} variants can be demonstrated in erythrocyte populations by the fluorescent antibody method. This may be of value in genetic studies aiming at the detection of isoantigenic variants in populations of somatic cells, and in cases of natural or artificial chimerism.

The ability of intact living mouse cells to absorb H-2 isoantibodies was interpreted to mean (12) that isoantigens are localized at the cell membrane, while the immunizing ability of nuclear preparations was taken as suggestive of a nuclear localization (3). After having studied the intracellular distribution of H-2 isoantigens capable of provoking immunological enhancement, Kandutsch (19) came to the conclusion that the wide distribution of enhancing activity in all the particulate fractions isolated by him was in general agreement with the hypothesis that the enhancing antigens were distributed throughout the membranes of the cells. This would be in good agreement with the present results. Billingham *et al.* and also Oth *et al.* (27) chose the induction of homograft immunity as their criterion of antigenicity while Kandutsch studied the enhancement of tumor homografts. Since enhancement depends on the formation of humoral antibodies (18), Kandutsch actually deals with antigens capable of provoking the formation of humoral antibodies. According to Medawar (22) isoantigens can be divided into T-antigens that induce transplantation immunity and H-antigens that stimulate the formation of humoral antibodies. By definition, both the fluorescent antibody method and the enhancement criterion of Kandutsch are concerned with the H-antigens. The parallel findings in these two studies as opposed to the conclusion of Billingham *et al.* and Oth *et al.* may be taken as a possible indication of a difference in the localization of these two kinds of antigens. It has to be pointed out, however, that comparisons of this type, indicating differences in the localization of isoantigens, must be evaluated with caution, in view of the fact that different experimental procedures were used and there was considerable variation in the criteria. Negative results with the fluorescent antibody method do not necessarily indicate the absence of antigenic receptors, since the sensitivity of this method is limited by the concentration of antigens rather than by their absolute amount (23). On the other hand, results from extraction experiments with different subcellular fractions may be misleading since contamination of the fractions cannot be excluded at present (19).

SUMMARY

The fluorescent antibody technique has been applied for the demonstration of mouse isoantigens at the cellular level. Specific reactions were obtained by the indirect or "sandwich" technique with a variety of living normal and neoplastic cells. Isoantigens of the H-2 system and of other systems could be demonstrated as well and appeared to be localized at the cell membrane. As far as the H-2 system was concerned, the membrane localization could be confirmed on histological sections.

Different types of non-specific staining reactions have been identified and described. Pinocytosis and cell injury led to such reactions that were morphologically distinguishable from the specific "ring" reaction and as far as pinocytosis is concerned, could be easily avoided by reducing the incubation time. In addition, a non-specific staining reaction morphologically indistinguishable from the specific "ring" reaction could be seen in a small proportion of bone marrow and lymph node cells but in no other cell type studied. The possible nature of this reaction is discussed.

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EXPLANATION OF PLATES

PLATE 40

FIG. 1. Tissue section of solid TA3 tumor treated with C57L anti-A serum followed by a mixture of absorbed fluorescein-conjugated rabbit anti-mouse globulin and unabsorbed, rhodamine-conjugated bovine albumin. Intercellular boundaries are outlined by green fluorescence. Nuclei unstained. × 80, exposure time 5 minutes.

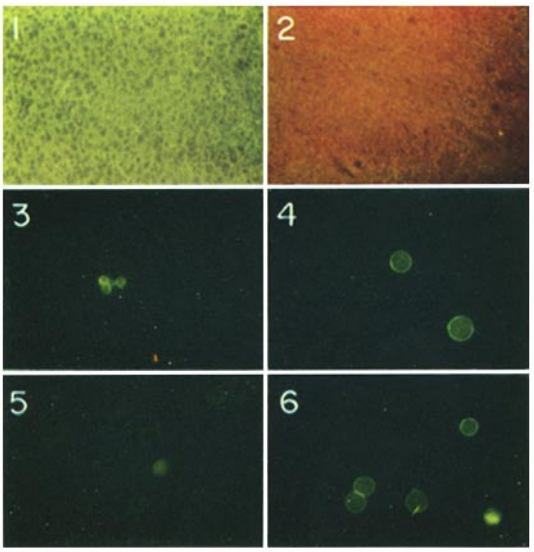
FIG. 2. Same as Fig. 1 but treated with normal mouse serum instead of antiserum. Absence of green fluorescence. Homogeneous red staining due to rhodamine-conjugated bovine albumin. × 80, exposure time 5 minutes.

FIG. 3. Living A lymph node cells treated with C57L anti-A isoantiserum in suspension followed by fluorescent rabbit anti-mouse globulin. "Ring" reaction in all cells. × 200, exposure time 4 minutes.

FIG. 4. Living MC1M ascites tumor cells treated with A.CA anti-C3H serum followed by fluorescent rabbit anti-mouse globulin. The suspension was fixed in 95 per cent ethanol after completion of staining. Typical "ring" reaction in all cells. × 200, exposure time 4 minutes.

FIG. 5. Injury reaction. Living TA3 ascites tumor cells treated with normal mouse serum in suspension followed by fluorescent rabbit anti-mouse globulin. One diffusely stained injured cell is seen among ten unstained living cells. The latter are difficult to observe owing to their weak autofluorescence. × 200, exposure time 5 minutes.

FIG. 6. Living MC1M ascites tumor cells treated in suspension with A.CA anti-C3H antiserum followed by fluorescent rabbit anti-mouse serum. Fixation in 95 per cent ethanol after staining. Four cells show the "ring" reaction, one cell shows an unusually strong injury reaction. × 200, exposure time 4 minutes.

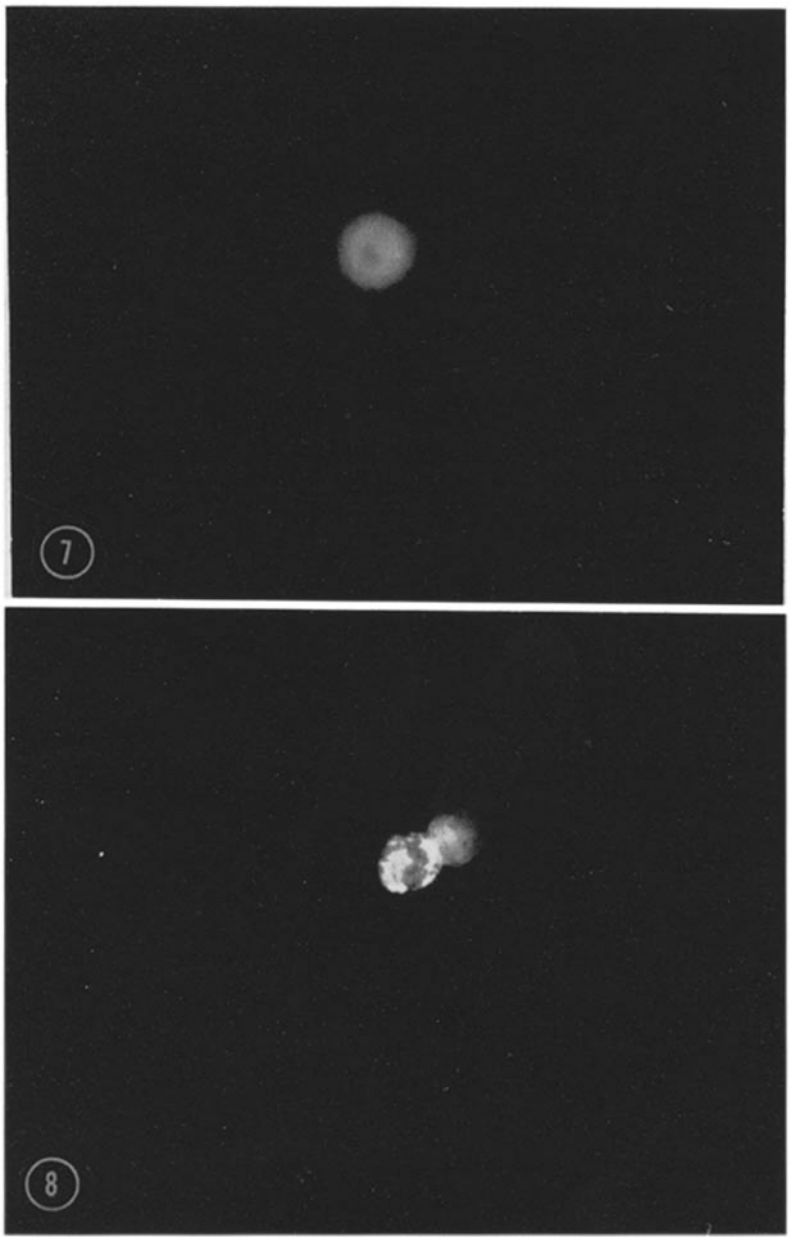


(Möller: Mouse isoantigens)

PLATE 41

FIG. 7. Living MC1M ascites tumor cell suspension treated with normal serum followed by rabbit anti-mouse globulin. The figure shows the injury reaction with diffuse fluorescence. × 500, exposure time 4 minutes.

FIG. 8. Living TA3 ascites tumor cells incubated for 2 hours at 37°C with fluorescent rabbit anti-mouse globulin. One cells shows pinocytosis with fluorescent vacuoles localized in the cytoplasm, the other exhibits the injury reaction. The vacuoles in the cell showing pinocytosis are not clearly distinguishable in the figure because of difficulties in getting all the vacuoles in focus. × 500, exposure time 3 minutes.

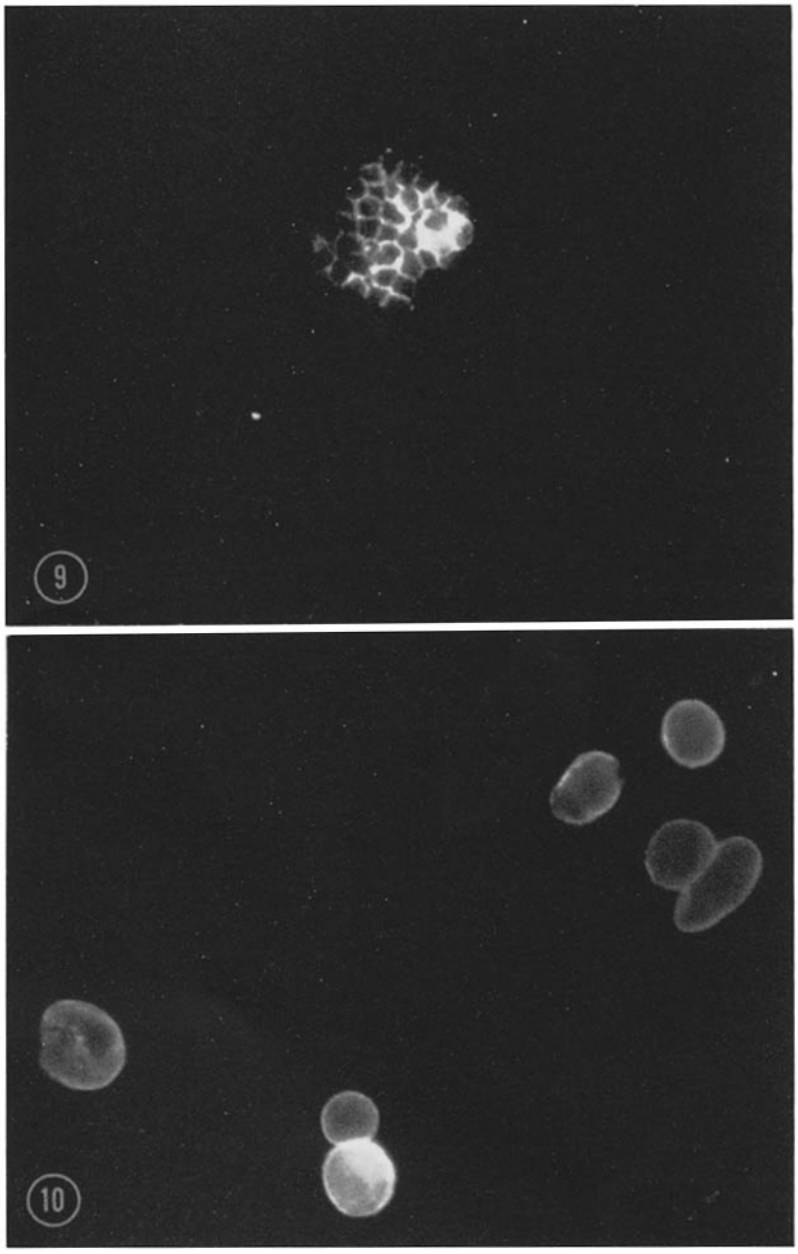


(Möller: Mouse isoantigens)

PLATE 42

FIG. 9. Strain A erythrocytes treated with C57L anti-A serum followed by fluorescent rabbit anti-mouse serum. The cells are agglutinated and show the "ring" reaction. × 500, exposure time 5 minutes.

FIG. 10. Living epithelial cells of an A x C3H F_1 hybrid mouse, treated in suspension with C57L anti-A serum, followed by fluorescent rabbit anti-mouse serum. All cells show the "ring" reaction of different intensity. One cell is overexposed. × 500, exposure time 4 minutes.

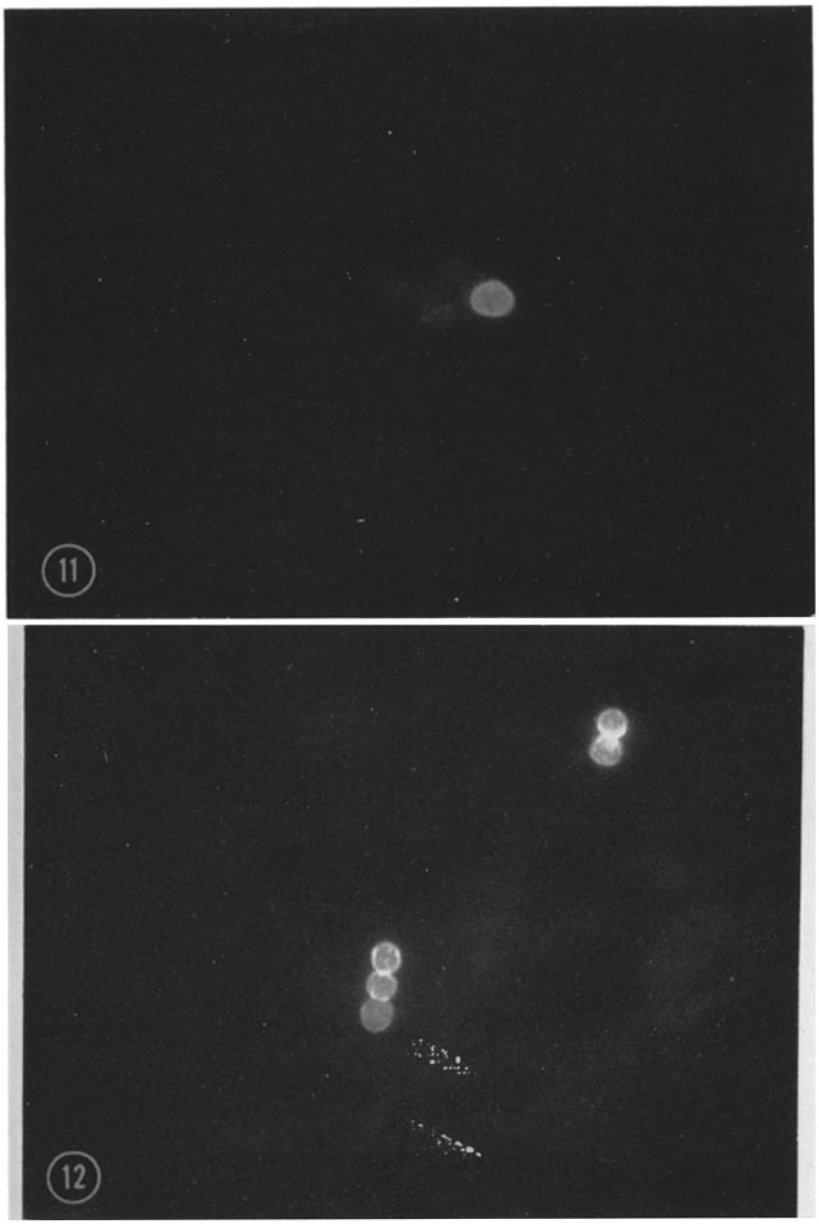


(Möller: Mouse isoantigens)

PLATE 43

FIG. 11. The appearance of a cell suspension of strain A lymph node cells treated in suspension with normal serum followed by fluorescent rabbit anti-mouse globulin. One cell shows the "ring" reaction, six cells are completely unstained. × 500, exposure time 4 minutes.

FIG. 12. Strain C57Bl lymph node cells treated in suspension with A.BY anti-5L1 serum followed by fluorescent rabbit anti-mouse serum. All cells in this figure show the "ring" reaction. × 500, exposure time 5 minutes.



(Möller: Mouse isoantigens)