Further Studies on Natural Antisheep Agglutinins in Mice of Inbred Strains*†

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INTRODUCTION

In earlier work, natural and immune hemoantibodies were studied in mice of inbred strains with low and high spontaneous tumor incidence (5, 6). A difference between different strains was observed in that natural antisheep agglutinins occurred commonly in C57 black mice, whereas these antibodies were found much less frequently in five other strains (C3H, dba, Marsh-albino, B alb C, Akm); as a rule their titers were, when present, considerably lower in these strains. A number of questions arose in connection with this difference in hemoantibodies by which the C57 black strain is apparently set apart from other inbred strains. The present report deals with attempts to find answers to some of these questions, particularly with regard to the origin and nature of the agglutinins.

EXPERIMENTAL

The technics used were identical with those reported previously (6).

Antigenic structure of mouse tissues and antisheep agglutinins.—We have previously considered the possibility that the presence or absence of natural antisheep agglutinins, as found in the different mouse strains, may be the expression of antigenic differences existing in these strains (6). That is, the situation might be similar to the relationship between isoagglutinins and isoagglutinogens in man: the presence of the antigen excludes the presence of the homologous antibody. Antigenic differences between inbred mouse strains were demonstrated by Gorer (11, 12), who reported the finding of certain antigens in red blood cells in some, and not in other strains. Recent work by Maculla (16) was concerned with antigenic analysis of mouse tissues, but without consideration of possible strain differences.

We attempted to answer the following questions: Does the absence of natural antisheep agglutinins depend on the presence of the homologous antigen in red blood cells or tissues, and vice versa? Or are there merely quantitative differences of the same type?

In order to test this assumption, pooled serum of C57 blacks containing natural antisheep agglutinins was absorbed with tissues and red cells of mice of different strains. Tissues of C3H and dba animals were taken from animals without antisheep agglutinins in their serum, while tissues of C57 blacks were derived from animals with antisheep agglutinin titers of at least 1:8. Absorptions of the serum with fresh and boiled sheep cells, guinea-pig kidney, and kaolin suspensions served as controls. Two parts of 50 per cent suspensions of the antigens were mixed with 1 part of serum. The mixtures were incubated, with repeated shaking, for 1 hour at room temperature, and then the supernatant was separated for testing. The results of one such experiment are summarized in Table 1.

TABLE 1
RESULTS OF ABSORPTION OF POOLED C57 BLACK SERUM

	Titer
Unabsorbed	16
After absorption with:	
Fresh sheep cells	0
Boiled sheep cells	16
Guinea-pig kidney	8
Kaolin	16
Kidney, C3H	16
Kidney, dba	16
Kidney, C57 black	16
Spleen, C3H	8
Spleen, dba	16
Spleen, C57 black	16

With the exception of fresh sheep cells, none of the antigens removed significant amounts of the antisheep agglutinins, and, in particular, no differences

* Presented at the Forty-first Annual Meeting of the American Association for Cancer Research, Atlantic City, New Jersey, April 18, 1950.

† This investigation was supported (in part) by a research grant from the National Cancer Institute, National Institute of Health, Public Health Service.

Received for publication May 6, 1950.

were noted in the absorbing ability of mouse tissues derived from different strains. Identical results were obtained in absorptions with liver and erythrocyte suspensions of the three strains. The failure of guinea-pig kidney and of boiled sheep cells to remove the antibodies confirms our earlier findings (5) that the natural antisheep agglutinins in mice are not heterophilic antibodies of the Forssman type.

Further studies of the antigenic properties of mouse tissues included investigation of their ability (*a*) to produce antibodies in suitable experimental animals and (*b*) to react *in vitro* with the antibodies so produced.

Several years ago Brown (3) reported the production of antisheep hemolysins in rabbits by the injection of heart, liver, and muscle of mice. While the publication did not contain any reference to the strains used in this work, the author kindly made available to us unpublished data[1] which showed that the antibody production and the results of absorption appeared in no way dependent on the strain which was utilized (C3H, A, C alb, C57 brown, C57 black, dba, NB, Y, and C58). Antisheep agglutinins were not included in the investigation. Red cells of mice were found incapable of producing antisheep hemolysins. Brown concluded from his absorption experiments and other findings that the antigen in mouse tissues responsible for the production of antisheep hemolysins was not identical with the Forssman antigen.

The following antigens were used in our experiments: spleen, kidney, tumor tissue, liver, and erythrocytes. These tissues were derived from strains C3H, dba, and C57 black. The tumors used were: spontaneous mammary carcinoma of C3H, transplanted mammary carcinoma of dba, and methylcholanthrene-induced sarcoma of C57 black. As a rule, two rabbits were injected with each antigen. In the experiments with erythrocytes, liver, and kidney, suspensions of pooled tissues (or erythrocytes) of the respective strains were used, without regard to the titer of natural antisheep agglutinins in the animals from which the antigen was derived. In the experiments with spleen and tumor tissue, the antigens were derived from animals whose blood had been previously tested for antisheep agglutinins. For preparation of tissue suspensions from strains C3H and dba, animals which showed no natural antisheep agglutinins in the blood were selected, while tissues for C57 black antigens were derived from animals with titers of antisheep agglutinins of at least 1:8. In the absorption experiments, to be mentioned later, only tissues from animals tested for antibodies and selected in the manner just indicated were used.

[1] Personal communication.

The red blood cells were injected as 33 per cent suspensions, by volume, and all other antigens as 3.5 per cent suspensions by weight; intravenous injections were given every third or fourth day, with the dosage increasing from 0.25 cc. to 1.0 cc. One week after the sixth injection, samples of the rabbit serum were tested for antibodies. If the titers were low, a second course was given before the final bleeding. In some instances, further injections were given as "boosters" to supplement the supply of serum.

Table 2 shows the titers of antisheep agglutinins and hemolysins which were found in the rabbits after injection of spleen, kidney, and tumor tissue derived from the different strains. The results with erythrocytes and liver suspensions are not tabulated. Erythrocytes of the three strains tested did not elicit development of antisheep agglutinins or hemolysins in rabbits. The liver suspensions produced only low titers of antisheep hemolysins and no antisheep agglutinins, regardless of strain derivation.

Injections of kidney, spleen, and tumor tissue were followed by greater antibody response, with the titers increasing in the order mentioned. The titers of hemolysins were considerably higher than those of agglutinins, except in response to spleen. Furthermore, the agglutinins proved less stable and disappeared on storage earlier than the hemolysins. No relationship was apparent between the titers of the antibodies and the rapidity with which they developed, on the one hand, and the strain of the animal from which the antigen was obtained, on the other.

Absorption experiments were carried out in the following way: 1 part of the anti-mouse rabbit serums was mixed with 2 parts of 50 per cent suspensions of boiled mouse tissues, and the supernatant was removed after 1-hour incubation at room temperature. Antigens derived from each strain were used to absorb each rabbit serum; parallel experiments with suspensions of fresh sheep cells, boiled sheep cells, boiled guinea-pig kidney suspensions, and kaolin served as controls for completeness of absorption or for unspecific absorption, respectively.

The antisheep hemolysins produced in rabbits by injection of mouse kidney were removed by boiled suspensions of mouse kidneys regardless of the strain from which the immunizing and the absorbing tissues were derived; the same results were obtained after absorption with suspensions of boiled guinea-pig kidney, and fresh and boiled red cells of sheep. Kaolin suspensions did not re-

move appreciable amounts of hemolysins.

Similar results were obtained with an antimouse spleen serum. Mouse kidney and spleen were used for the absorption. Splenic tissue removed the antibodies completely, whereas minimal traces of antibodies were noted after absorption with kidney. The kidneys of C3H animals were somewhat less efficient in removing the antibodies than kidneys of dba or C57 black mice. However, the differences were neither distinct nor consistent enough to warrant any conclusion.

Antimouse tumor serum produced in rabbits was absorbed with tumors, kidneys, and red blood cells of the different strains. The results are summarized in Table 3. Absorption with tumor tissue resulted in complete removal of the antibodies; absorption with kidneys showed less complete removal, and red blood cell suspensions failed to remove any antibodies. Again the absorption was not found to depend on the strain origin of the absorbing tissues.

In Table 4 experiments are summarized in

TABLE 2

HETEROPHILIC ANTIGEN IN MOUSE TISSUES

Mouse strain	Rabbit no.		Antibody titer: Before immunization, with spleen	Antibody titer: After immunization, with spleen	Rabbit no.	Antibody titer: Before immunization, with kidney	Antibody titer: After immunization, with kidney	Rabbit no.	Antibody titer: Before immunization, with tumor tissue*	Antibody titer: After immunization, with tumor tissue*
C3H	16	A†	1	512	22	1	8	28	16	16
	"	IH	5	5,120	"	40	1,280	"	80	5,120
	"	CH	0	640	"	10	160	"	10	1,280
"	17	A	2	512	23	1	16			
	"	IH	5	5,120	"	40	1,280			
	"	CH	1	640	"	10	320			
dba	14	A	0	256	20	4	32	32	2	32
	"	IH	5	2,560	"	80	640	"	40	5,120
	"	CH	1	320	"	20	160	"	5	2,560
"	15	A	1	1,024	21	1	2	33	2	32
	"	IH	5	5,120	"	10	640	"	160	5,120
	"	CH	0	2,560	"	5	160	"	40	1,280
C57 black	18	A	2	512	24	1	0	30	2	32
	"	IH	20	5,120	"	40	640	"	80	5,120
	"	CH	5	320	"	20	80	"	10	1,280
" "	19	A	0	512	25	1	8	31	2	128
	"	IH	20	5,120	"	20	1,280	"	80	10,240
	"	CH	0	640	"	5	320	"	5	5,120

* C3H—Spontaneous mammary carcinoma.
dba—Transplanted mammary carcinoma.
C57 black—Methylcholanthrene-induced sarcoma.

† A—Titer of antisheep agglutinins.
IH—Titer of incipient hemolysis.
CH—Titer of complete hemolysis.

TABLE 3

SEROLOGIC ANALYSIS OF ANTI-MOUSE TUMOR SERUM

Strain of immunizing antigen	Rabbit no.		Antibody titers: Unabsorbed	Kaolin	After absorption with: G.P. kidney, fresh and boiled sheep RBC	Tumor (C3H, dba, C57 bl.)	Kidney (C3H, dba, C57 bl.)	RBC C3H, C57 bl.
C3H	28	A*	8	8	0	0	2	8
	"	IH	1,280	640	0	0	10	2,560
	"	CH	160	160	0	0	0	160
dba	32	A	16	16	0	0	4	32
	"	IH	5,120	1,280	0	0	40	5,120
	"	CH	1,280	320	0	0	10	1,280
"	33	A	32	32	0	0	2	
	"	IH	1,280	1,280	0	0	40	
	"	CH	320	320	0	0	0	
C57 black	30	A	16	16	0	0	4	16
	"	IH	5,120	1,280	0	0	40	2,560
	"	CH	1,280	320	0	0	0	640
" "	31	A	32	32	0	0	8	
	"	IH	10,240	2,560	0	0	160	
	"	CH	2,560	1,280	0	0	10	

* A—Titer of antisheep agglutinins.
IH—Titer of incipient hemolysis.
CH—Titer of complete hemolysis.

which two immune serums were used: (*a*) that of guinea pigs injected with red cells of sheep; (*b*) that of rabbits injected with boiled suspension of guinea pig kidney. The first serum is a true isophilic serum (serum which lacks Forssman-type antibodies because of the presence of Forssman antigen in the tissues of the guinea pig). The antibodies for sheep cells present in the second serum, on the other hand, are true heterophilic antibodies, because the tissues of the rabbit lack Forssman antigen and the antigen used in immunization is the prototype Forssman antigen. Only fresh red cells of sheep absorbed the antibodies from the isophilic immune serum. On the other hand, all the known carriers of the Forssman antigen and the kidney and tumor suspensions of mice removed the antibodies from the heterophilic immune serum, completely or in large amounts. Rabbit kidney, known to be free of Forssman antigen, lacked absorbing ability, as did the nonspecific control suspension of kaolin. Mouse kidney was a slightly less potent absorbing agent than was mouse tumor tissue; this finding is in good agreement with their respective immunizing properties. There was no difference in the behavior of kidney or tumor tissue depending on strain derivation.

TABLE 4

ANTIGENIC ANALYSIS OF MOUSE TISSUES

	Antibodies					
	Isophilic*			Heterophilic†		
	A	IH	CH	A	IH	CH
Unabsorbed	320	2560	320	40	2560	640
After absorption with:						
Fresh sheep RBC	0	0	0	0	0	0
Boiled sheep RBC	160	1,280	160	0	0	0
Guinea-pig kidney	320	1,280	160	0	0	0
Rabbit kidney	160	2,560	320	20	2,560	320
Kaolin	320	1,280	320	20	1,280	320
Mouse kidney, C3H	160	640	160	20	160	40
Mouse kidney, dba	160	640	160	0	160	40
Mouse kidney, C57 bl.	160	640	160	0	80	0
Mouse tumor, C3H	160	640	160	0	40	0
Mouse tumor, dba	160	640	160	40	80	0
Mouse tumor, C57 bl.	160	640	160	0	0	0

* Antisheep RBC guinea-pig serum.
† Anti-guinea pig kidney rabbit serum.

Antisheep agglutinins in additional inbred strains.—Since our last report (6), the total number of strains examined and of animals within the strains has increased. Table 5 presents a summary of the results now available. The largest increase in animals tested has been in strain C57 black. The percentage of zero titers in this group has increased from 3.5 to 9.8, while the percentage of titers of 16 and higher has decreased from 55.4 to 43.9. As an explanation it may be suggested that a study of genetic factors which is under way showed that there are some "sublines" of C57 black mice with considerably higher incidence of zero titers and with few high titers.

In addition to the six strains previously tested, two more have been examined, C57 brown, subline cd, and strain I.[2] Both strains showed a low incidence of natural antisheep agglutinins.

Age and antisheep agglutinins.—In animals tested in previous work (5, 6), no relationship between presence or titer of antisheep agglutinins and age was observed. The age of these animals ranged from 10 weeks to 20 months, with but few of them younger than 3 months. Subsequent work showed that animals younger than 12 weeks, regardless of strain, possessed antisheep agglutinins less frequently than older animals. For this reason, a systematic study of the relation between age and antisheep agglutinins was undertaken. Eight age groups ranging from 5 to 12 weeks, of C57 black mice, born in this laboratory,[3] were tested. Each group consisted of 30 animals, with both sexes approximately evenly distributed. More than 80 per cent (25 of 30) of the animals in the youngest age group (5 weeks) had no antibodies, even in undiluted serum. The highest titer (1:8) was found in only one animal. The incidence of antibodies

TABLE 5

ANTISHEEP AGGLUTININS IN INBRED STRAINS OF MICE

Strain	No. of animals tested	Agglutinins absent		Agglutinins present		Titers of 16 and higher	
		(No.)	(Per cent)	(No.)	(Per cent)	(No.)	(Per cent)
C57 black	326	32	9.8	294	90.2	143	43.9
C3H	122	84	68.9	38	31.1	0	0
dba	186	84	45.2	102	54.8	9	4.8
Marsh-albino	56	21	37.5	35	62.5	1	1.8
B alb C	60	21	35.0	39	65.0	0	0
Akm	87	61	70.1	26	29.9	0	0
C57 brown	91	53	58.2	38	41.8	1	1.1
I	81	67	82.7	14	17.3	0	0

[2] Strain I animals were kindly provided by Dr. John J. Bittner, Division of Cancer Biology, University of Minnesota. Strain C57 brown animals were purchased from Jackson Memorial Laboratory, Bar Harbor, Maine.

[3] The colony was bred from stock received in 1946 from Dr. A. Tannenbaum, Michael Reese Hospital, Chicago, Illinois.

was similar in the animals 6 and 7 weeks old, but changed significantly in the eighth week, when the number of animals without antibodies dropped to 15 (50 per cent); titers of 1:8 or higher were found in five animals, and 1:32 was the highest titer observed. This trend continued steadily in higher age groups, and it reached the minimum of 33 per cent of animals without antibodies and the maximum titer of 1:128 (in one animal) in the age group of 12 weeks. The comparatively small number of animals comprising each of the age groups makes it understandable that fluctuations in the antibody distribution were observed between 5 and 12 weeks, but it is apparent that around the age of 8–10 weeks there is a definite increase in the incidence of agglutinins which continues during the next weeks. It is also noteworthy that higher titers of the antibodies—1:32 and more—were found first in mice 8 weeks old and that the number of animals with these higher agglutinin levels increased during the subsequent weeks.

DISCUSSION

The experiments failed to detect antigenic differences which could account for the differences in the incidence of natural antisheep agglutinins in the strains tested. We realize that these negative findings cannot be considered to prove conclusively the absence of such antigenic differences. It is conceivable that by using whole tissues as antigens, as was done by us, differences could be masked which might become apparent if lipid fractions were to be used instead, since it is known that antigens of the Forssman type are fat-soluble. This problem is being investigated.

These results confirm previous reports on the presence of Forssman antigen in tissues of mice. Some of our results are not in agreement with those recently reported by Brown (3). The discrepancies will be discussed in a separate presentation.

The results obtained with tumor tissues, both in the immunization and the absorption experiments, showed them to possess relatively large quantities of Forssman antigen. This is in good agreement with the old observation of Morgenroth and Bieling (17, 18) on heterophilic antigens in mouse carcinoma. This work was extended to other tumors more recently by Dmochowski (7–9). The three mouse tumors used by us did not differ from each other in their content of heterophilic antigen.

In our former communications it was pointed out that the C57 black strain, which is characterized by the high incidence of natural antisheep agglutinins, shows a low incidence of spontaneous mammary carcinoma (1 per cent or less) and of other, primarily nonepithelial tumors—10–20 per cent (13). It is not possible to correlate the incidence of antisheep agglutinins with the incidence of spontaneous mammary carcinoma, since strains Akm and B alb C, with low incidence of this tumor, were also found to have a low incidence of antisheep agglutinins. Strain Akm shows a high spontaneous incidence of leukemia, and in strain B alb C internal tumors are common (13). In the C57 brown strain, subline cd, the females are reported to show a medium incidence of spontaneous mammary carcinoma (13). The literature on strain I was reviewed recently by Andervont (1). Spontaneous malignant tumors are rare among these animals; the gastric lesions, which occur in almost all animals, are considered benign, in spite of their peculiar behavior in transplantation experiments (2). Hence, it is apparent that no relationship can be shown to exist between the occurrence of natural antisheep agglutinins and the tendency to development of spontaneous tumors in the individual strains.

The absence of a relationship between the natural antisheep agglutinins and the presence of the milk agent was reported.[4]

The observations in young mice suggest that testing for antisheep agglutinins should not be done in animals less than 12 weeks old if one wants to establish the incidence of the antibodies in a particular strain. With the exception of the few animals tested some time ago, we have followed this rule.

The findings are in accord with previously recorded observations that natural hemoantibodies develop postnatally, a phenomenon which was designated by Hirszfeld (14) as "serologic maturation." This phenomenon is well known from studies on human isoagglutinins (4, 20, 21). Similar observations on heteroagglutinins in chicks have been recently reviewed and confirmed (15).

The gradual postnatal development of antisheep agglutinins does not offer any clue as to why they are found so much more frequently in C57 black mice, as compared with mice of other strains. It has been shown that heterophilic antibodies may owe their formation to antigens introduced in the food (10, 19). It may be that natural antibodies have a similar origin. Such an assumption could have hardly any bearing on the present problem, since C57 black animals and animals of the other strains received the same stock diet (Rockland mouse diet). It may also be that natural

[4] Davidsohn, I., Stern, K., and Bittner, J. J. Milk Agent and Natural Antisheep Agglutinins in Mice of Inbred Strains. Proc. Soc. Exper. Biol. & Med. (in press).

agglutinins develop in response to enteral immunization by intestinal saprophytes. Differences in the composition of the intestinal flora of various strains have not to our knowledge been studied.

SUMMARY

1. Natural antisheep agglutinins have been studied thus far in strains C57 black, C3H, dba, Marsh-albino, B alb C, Akm, C57 brown, and I.

2. The antisheep agglutinin in mice is not of the Forssman type.

3. Antigen of Forssman type was found in normal and neoplastic tissues of C3H, dba, and C57 black mice, independent of strain. The antigen was not present in the erythrocytes.

4. No relationship was noted between presence and titer of natural antisheep agglutinins and the incidence of spontaneous tumors in the strains studied.

5. The incidence and titer of antisheep agglutinin in C57 black mice showed a progressive rise from the fifth to the twelfth weeks of life.

REFERENCES

1. Andervont, H. B. Spontaneous Lesion of Stomach in Strain I Mice. J. Nat. Cancer Inst., **16**:405–6, 1949.
2. Andervont, H. B., and Shimkin, M. B. Intrapulmonary Transplantation of the Adenomatous Gastric Lesion of Strain I Mice. J. Nat. Cancer Inst., **2**:151–55, 1941.
3. Brown, G. C. Antigenic Properties of Mouse Tissues. J. Immunol., **46**:325–32, 1943.
4. Davidsohn, I. Isoagglutinin Titers in Serum Disease, in Leukemias, in Infectious Mononucleosis, and after Blood Transfusions. Am. J. Clin. Path., **8**:179–96, 1938.
5. Davidsohn, I., and Stern, K. Hemagglutinins in the Serum of Mice of Low and High Mammary Tumor Strains. Proc. Soc. Exper. Biol. & Med., **70**:142–46, 1949.
6. ———. Natural and Immune Antibodies in Mice of Low and High Tumor Strains. Cancer Research, **9**:426–35, 1949.
7. Dmochowski, L. Über die serologische Spezifizität der tierischen Tumor und Eiterantigene. Ztschr. f. Immunitätsforsch. u. exper. Therap., **93**:311–30, 1938.
8. ———. Investigations on the Serological Specificity of Experimental Tumors. Am. J. Cancer, **37**:252–64, 1939.
9. Dmochowski, L., and Pryszczynski, A. Über die Grundlagen der serologisch fassbaren Veränderungen in den Organen beim Krebs und bei der Tuberkulose. Ztschr. f. Immunitätsforsch. u. exper. Therap., **93**:331–45, 1938.
10. Forssman, J. Some Experiments on Origin of "Normal" F-Hemolysin in the Rabbits and Occurrence of F-Antigen in Plants. Acta path. et microbiol. Scand., **23**:145–57, 1946.
11. Gorer, P. A. The Detection of a Hereditary Antigenic Difference in the Blood of Mice by Means of Human Group A Serum. J. Genetics, **32**:17–31, 1936.
12. ———. The Detection of Antigenic Differences in Mouse Erythrocytes by the Employment of Immune Sera. Brit. J. Exper. Path., **17**:42–50, 1936.
13. Heston, W. E. Genetics of Mammary Tumors in Mice. A Symposium on Mammary Tumors in Mice, AAAS, p. 61, Table I, 1945.
14. Hirszfeld, L. Konstitutionsserologie und Blutgruppenforschung. Julius Springer: Berlin, 1928.
15. Kershaw, B. B. Studies on the Origin and Development of Normal Hemagglutinins. Yale J. Biol. & Med., **21**:463–80, 1949.
16. Maculla, E. S. The Immunochemistry of Mouse Tissue Components. Yale J. Biol. & Med., **20**:299–314, 343–68, 465–72, 1948.
17. Morgenroth, J. Hämolytische Versuche. Berlin. klin. Wchnschr., **50**:561, 1913.
18. Morgenroth, J., and Bieling, R. Amboceptoren und Receptoren (zugleich ein Beitrag zur Kenntnis der Geschwulstimmunität). Biochem. Ztschr., **68**:85–117, 1915.
19. Powell, H. M. Immunization with Heterophile Antigen when Given by Mouth. Am. J. Hyg., **5**:228–39, 1925.
20. Schiff, F., and Mendlowicz, L. Quantitative Untersuchungen über Isoagglutinine mit besonderer Berücksichtigung der Leukämie. Ztschr. f. Immunitätsforsch. u. exper. Therap., **48**:1–22, 1926.
21. Thomsen, O., and Kettel, K. Die Stärke der menschlichen Isoagglutinine und entsprechenden Blutkörperchenrezeptoren in verschiedenen Lebensaltern. Ztschr. f. Immunitätsforsch. u. exper. Therap., **63**:67–93, 1929.