INTRODUCTION

The present writer (8-10) has advanced the theory that the genes determining the fate of transplanted tissues are identical with those determining the presence of iso-antigenic differences. Such differences are most easily studied in erythrocytes. Strong's A strain happens to possess two iso-antigens that are shared by the erythrocytes and a number of fixed tissues, including all neoplastic tissues studied so far. These antigens have been called antigens I and II, the latter being the more potent. The best antibody producers found to date have been members of Little's C57 black strain.

A spindle cell sarcoma and a myeloblastic leukemia arising in the A strain have been tested on hybrids between these two strains. In each case it appeared that 2 dominant genes were essential for growth, one of which appeared to be identical with the gene for antigen II. Sera for the detection of antigen I are difficult to obtain and were not available for the genetical investigations. Mice in which the tumors had regressed produced isoagglutinins; in most cases these could be identified as "anti-II," but certain sera contained an additional agglutinin that reacted with the cells of mice of the CBA strain, which shares antigen I with the A strain but lacks II. It seems likely, therefore, that both neoplasms contain antigens I and II (9). Agglutinins may also be produced by inoculation of blood or normal tissues, neoplastic tissues giving the better response, partly perhaps because of their greater proliferative power, but possibly because they contain more of the pertinent antigens than normal tissues (9, 10).

The results summarized in the preceding paragraph were obtained with animals that had been inoculated once. The response to hyper-immunization was studied principally in connection with leukemia. Here it appeared that following two or more injections, the hemagglutinins disappeared. However, sera that were apparently free from agglutinins could protect mice of the A strain against inoculation with leukemic cells, as could those from which the agglutinins had been removed by absorption. It was deduced that mice produced two sorts of antibody, agglutinins and protective antibodies. The latter could be partially absorbed by red cells but far less readily than by leukemic cells, indeed the absorption by the former was difficult to detect. However, the agglutinins are far more easily absorbed by neoplastic cells than by erythrocytes and the conclusion was reached that whilst the two antibodies were qualitatively distinct, they were both directed against the same antigens (10).

At this point the investigations were interrupted by the war. In the meantime, important advances have been made in the study of iso-immunization to the rhesus antigens in man. Here again it appears that two qualitatively distinct antibodies may be formed, ordinary agglutinins and partial or blocking antibodies (11, 13). The latter need special methods for detection in vitro, reference to which will be made below, but they appear to be of greater functional significance and are sometimes referred to as the mature type of Rh antibody (3, 15). This situation shows interesting analogies to that just described for mice and we, therefore, decided to see whether such antibodies could be detected in this species.

MATERIALS AND METHODS

The Roscoe B. Jackson Memorial Laboratory had two A strain tumors available, known as 15091a and C1300. The former has been described as a spindle cell mammary carcinoma. It is a pleomorphic anaplastic growth with fairly numerous giant cells. The latter is a round cell tumor, possibly a neuroblastoma. Of the two, 15091a is much more virulent for heterologous pure strains, causing a high mortality in many of them. In the
C57 black mice it seldom causes death but far larger masses are usually produced than is the case with C1300. They were inoculated in the ordinary way by trocar and canula.

Previous experience has shown that the agglutination tests must be performed in tubes and that in reading, care must be taken to avoid too much force in breaking up the cellular deposit. The author (7, 9) has found the following method suitable: About 0.05 cc. of serum dilution and 1 per cent cell suspensions are set up in dwarf test tubes and incubated for an hour or more at 37°C. The tubes are then centrifuged for 2 minutes at about 700 r.p.m. (it is important that spinning should not be too violent or too long). The readings are then made by gently pumping the supernatant back and forth with a capillary pipette. In the absence of agglutination the cells come away as an even cloud, sometimes leaving a small wisps of cells attached to the tube. After pumping 4 times most of the deposit should be resuspended.

If there is feeble agglutination, the deposit may be easily resuspended but has a granular appearance. As the strength of the reaction increases, the deposit breaks up into lumps of increasing size and often comes off as a solid pellet. There is also a tendency for agglutinated cells to stick to the glass, and this in itself is often a helpful criterion. There is only one real source of confusion in microscopical examination. In controls one may often see a few large clumps of cells. If the slide is gently tilted these will break up, the cells coming away singly. If they come away in small clumps there is some agglutination.

When doing tests where the final concentration of serum or plasma is relatively low, it is not necessary to wash the cells. If the final concentration of serum or plasma is high (say 50 per cent or over) washing is essential. It has been found most satisfactory to perform the washing with the 1 per cent suspensions. These are centrifuged at about 700 r.p.m. Two such washings are sufficient although 3 have sometimes been used. If the cells are not washed but suspended in pure serum, the whole mixture may clot, or partial clotting may occur, closely mimicking agglutination.

For the detection of partial antibodies in man the following technics have been used: the blocking test (11, 13), the conglutinin test of Wiener (14), the anti-globulin test of Coombs, Mourant and Race (2), the open slide (4) and albumin tests of Diamond and his co-workers (6). All of these have been tested. The last two seem inapplicable to mouse antisera. Human albumin was used and found to be lytic for mouse cells in saline; the lysin was inhibited by serum, but it did not bring about agglutination by blocking antibodies. The Coombs test gave some suggestive results and will have to be retested with more potent sera.

The technic of the blocking test will be described below. Wiener's conglutinin test depends upon the performance of all operations in compatible serum or plasma. This has been used successfully although the results are slightly different from those obtained with human sera. In this case it has not been found essential to avoid the slightest dilution of serum. When testing some sera the cells have been suspended in saline and all antibody dilutions done in compatible serum. This gives a final concentration of serum of 50 per cent. The most satisfactory system for general use has been with the serum dilutions as before but with the cells suspended in 50 per cent serum, giving a final concentration of 75 per cent serum. Where the standard method has been used, it is referred to simply as "saline." Except where stated to the contrary, pooled sera from 6 mice were titrated.

RESULTS

The Detection of Antibodies in Normal Serum

Up to the present it has been generally agreed that mice do not possess natural iso-agglutinins. It was, therefore, surprising to find that sera of C57 blacks would often cause agglutination of A strain erythrocytes. At first it was thought that this might be non-specific agglutination due to some undetected technical error. This is not the case since C57 black cells are not agglutinated. Originally the discovery was made with pooled sera from 6 or more mice. Later it was found that about 20 per cent of blacks gave some agglutination. Usually the reactions were feeble and could only be detected if the cells were suspended in the sera. However, in one pool strong agglutination was found. The antibody reacted with the cells of strains A, C3H and dba, but not with those of Bagg albino C, or the C57 blacks.

At the present time it is not possible to say what stimulus elicits these iso-antibodies. They have been found in both sexes, but it is possible that fetuses containing the pertinent antigens may sometimes stimulate their formation. This aspect of the problem would probably repay investigation.

The natural iso-antibodies can easily be absorbed, but it has been found more convenient to use the sera of strain A or C3H animals as a vehicle, and this has been done in the experiments to be reported below.

1 This has been found to be true of Swiss mice also.
THE ANTIBODY RESPONSE TO BLOOD

It is well known that no two tumors are exactly alike and that a given tumor may undergo variation. Of the normal tissues, blood has many advantages and a few preliminary experiments were therefore performed in order to see if the results were of the same kind as those obtained previously. Citrated whole blood was inoculated intraperitoneally, allowing for the dilution about 0.25 cc. were given at each inoculation. In the first experiment (see Table I) the inoculations were given at weekly intervals. The mice were bled after the first, second and fourth inoculations. In the second experiment the mice were injected twice a week until about 1.0 cc. had been given, when they were bled. They were rested for three weeks and then given two inoculations of 0.5 cc. within a week. The objective of the second experiment was to keep the mice constantly flooded with antigen as probably occurs with a tumor that proliferates and then regresses.

The first series is perhaps the most instructive. It will be seen that following the second 0.25 cc. there is an apparent drop in the titer of antibodies, to be followed by a rise in titer after 1.0 cc. had been given. Comparison with the second series shows that the response is less good when 1.0 cc. is given in concentrated dosage over 2 weeks than when the course is spread over a month.

Normal serum did not enhance agglutination to any great extent in these experiments. It will be noticed that there is a suggestion of a pro-zone with the hyper-immune sera.

THE RESPONSE TO TUMOR C1300

As is the case with blood, the spacing of the inoculations has an influence on the result. A short, intensive course of inoculations gave a lower titer than when they were spread out (see last titer, Table II). As a rule it is best to allow about 2 weeks to elapse following the first inoculation and give the subsequent ones whenever the tumor is ready to transfer. Probably about 10 day intervals are the best, but one can get almost the same result if the animals are rested a month or more and then given a booster inoculation.

The first four sera shown in Table II were all from the same group of animals. This is the type of response that may be taken as typical. Here again we see an apparent disappearance of agglutinins following a second inoculation. However, in this case titration in serum shows some agglutination with complete inhibition in the prozone. Following further inoculations there is a reappearance of agglutination in saline but the inhibition zone remains. This is almost invariably 1 tube shorter in serum. Further inoculations raise the titer and the inhibition zone tends to become shorter. In one group of animals it had disappeared after 6 inoculations. This is by no means always the case, however. An inhibition zone up to about 3/8 usually remains. Increasing the number of inoculations still further may sometimes cause an apparent drop in titer.

The fifth titration shows the effects of prolonging the interval between inoculation and bleeding. In that illustrated in Table II all agglutinins active in saline have disappeared, whilst in serum there is a fair titer with a marked inhibition zone. In another case there were apparently no antibodies at 21 days. However, this particular serum gave a strongly positive blocking test.

These experiments indicate that there are 3 types of antibody produced: ordinary agglutinins, agglutinins needing normal serum for their activity, and blocking antibodies.

THE RESPONSE TO TUMOR 15091a

This tumor has been studied in C57 brown as well as black mice. The latter are the more resistant but even here the response to primary inoculation is variable. The animals were usually inoculated

<table>
<thead>
<tr>
<th>Dose of</th>
<th>No. of</th>
<th>Dilution of serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood</td>
<td>days since</td>
<td>2</td>
</tr>
<tr>
<td>given,</td>
<td>last</td>
<td>+ + + +</td>
</tr>
<tr>
<td>cc.</td>
<td>inoculation</td>
<td>0.25</td>
</tr>
<tr>
<td>75% serum</td>
<td>75% serum</td>
<td>75% serum</td>
</tr>
<tr>
<td>1.0</td>
<td>7</td>
<td>Saline</td>
</tr>
<tr>
<td>75% serum</td>
<td>75% serum</td>
<td>75% serum</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>Saline</td>
</tr>
<tr>
<td>75% serum</td>
<td>75% serum</td>
<td>75% serum</td>
</tr>
</tbody>
</table>

*The meaning of the terms in this column are explained in the text. c = complete agglutination; a.c., almost complete agglutination; tr, trace, etc.
in groups of 12. Sometimes 2 or 3 of these died from the tumor. Thereafter 2 or 3 more groups were inoculated without a single death and with complete regression in about 2 weeks. This has been the common experience in recent months. The response to primary inoculation has been studied in the C57 blacks only. If there are persistent growths, one gets a serum with a marked inhibition zone as shown in the first entry serum in Table III. Following hyperimmunization in C57 blacks one does not get an apparent disappearance of antibodies, as occurred with the other two antigens. If the inoculations are given at very short

intervals, one may get an inhibition zone similar to that occurring with C1300 (fifth serum, Table III). If they are spaced out further one seldom gets a pro-zone at all. If there is one, there is only partial inhibition of agglutination.

One black mouse which was apparently dying of a large fungating growth, had blocking antibodies only. Dr. Snell was kind enough to give me some mice that had been rendered artificially susceptible by being inoculated with a lyophilized preparation of 15091a prior to the living tumor (12). These also were obviously in extremis. Their pooled sera gave the highest titer seen to date. It was over 16,000 in saline but only 4,096 in serum. This is an unusual finding.

It will be noticed that the ordinary agglutinins persist longer following inoculation with this growth than they do with C1300 (fourth serum, Table III).

Hyperimmunized C57 brown mice always give a pro-zone with this tumor. This was especially pronounced with serum of 2 animals with persistent growths. Two similar mice were tested individually. One had a smaller pro-zone than those shown in the table, with a titer of more than 4,096. The other showed blocking antibodies only.

### Antibodies Needing Enhancement With Normal Serum

As can be seen from Table I to III, in most cases agglutination is enhanced by normal serum. Two cases in which it is essential have already been awkward to store and cannot be used for experiments on the effects of heat. The enhancing factor (or factors) is thermostable, appearing to be undamaged by exposure to 60°C for 30 minutes. As a rule the serum is used almost as soon as it is available. One sample that had been stored in the frozen state for a month did not appear quite so good as a fresh sample. It is perfectly safe to lay in a week's supply at a time.

The amount of serum needed appears to depend upon the concentration of antibody. With natural antibodies one may get agglutination with 70 per cent. With feeble antigens such as the C3H carcinoma, the titer will be appreciably lower in 50 per cent serum than in 75 per cent serum. With some of the anti-C1300 sera, one may get some agglutination with as little as 10 per cent. It is obvious that the situation is different from that found by Wiener with human serum where it is essential to avoid any dilution. Furthermore, mouse serum will not cause agglutination with true blocking antibodies.

### The Detection of Blocking Antibodies

Blocking tests were first performed by Wiener and Race (11, 13) in connection with Rh sensitization, although Coca and Kelley had described essentially the same phenomenon in 1921 (1).

There are various possible modifications in the technic. In the present series of experiments two methods of titrating the sera were tried. In the first the test sera were diluted as in an ordinary

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**Table II: The Response of C57 Blacks to Tumor C1300**

| No. of injections | Day of bleeding | Titrated in | Saline | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum | 75% Serum |
|------------------|----------------|-------------|--------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 1                | 14             | c           | a.c.   | +        | +        | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         |
| 2                | 10             | c           | c      | ++       | ++       | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         |
| 3                | 23             | Saline      | a.c.   | +        | ++       | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         | +         |

**Notes**

First four titers, same group of animals.

Eighteen days between 1st and 2nd inoculation. Last 3 at 3 day intervals.
titration, incubated with red cells and a given quantity of agglutinin added to each tube. In the second case, undiluted immune sera were incubated together with red cells and varying quantities of agglutinin added. Examples of both of these are shown in Tables IV and V. Neither are ideal for titrating sera, although the latter appears preferable.

The first method has certain theoretical interest. The occurrence of a pro-zone suggests certain analogies with the inhibition due to antibody excess such as occurs with certain precipitating anti-sera from horses. It is difficult to check this with

<table>
<thead>
<tr>
<th>TABLE V: BLOCKING TEST</th>
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<tbody>
<tr>
<td>Blocking serum undiluted. Agglutinin varied.</td>
</tr>
<tr>
<td>Cells incubated in:</td>
</tr>
<tr>
<td>Saline</td>
</tr>
<tr>
<td>Normal A serum</td>
</tr>
<tr>
<td>Blocking serum</td>
</tr>
</tbody>
</table>

The blocking serum used here was anti-C15091a from an animal with a tumor. There was not enough serum for further tests.

This is done the pro-zone is invariably reduced, the reduction being greater with increasing amounts of added antibody, as is clearly shown in Table IV. This is the opposite of what would occur if the inhibition were due to antibody excess.

excess antigen, since with very heavy cell suspensions the results are difficult to read. With suspensions up to 10 per cent the pro-zone did not appear to be shortened nor could this be done by adding light suspensions of malignant cells. However, in both these cases it is possible that insufficient antigen was added. Therefore, it seemed easier to approach the matter from another angle and see the effect of adding excess antibody. If

<table>
<thead>
<tr>
<th>TABLE IV: BLOCKING TEST</th>
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<tbody>
<tr>
<td>Blocking serum diluted. Constant amount of agglutinin added.</td>
</tr>
<tr>
<td>Dilution of Blocking Serum</td>
</tr>
</tbody>
</table>

Agglutinin added

<table>
<thead>
<tr>
<th>Nil</th>
<th>Anti-C1300.</th>
<th>Anti-15091a.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>4-8</td>
<td>16</td>
</tr>
</tbody>
</table>

| Controls: Saline + anti-C1300 (4 units) | +++++ |
| Saline + anti-15091a (2 units) | + |

**The Effect of Storage on Sera**

It had been observed previously that the iso-agglutinins were largely destroyed by exposure to 60°C. for about 30 minutes. This observation has been confirmed with certain sera, but with others there was very little reduction in titer after such treatment. The other two types of antibody appear to be more thermostable. The sera were stored frozen solid, except in one case (the second serum in Table VI) which was left in the ordinary chamber of the ice box for 3 days. Apart from the fact that the agglutinins do not keep well, the behavior of any given serum is largely unpredictable. Sometimes there appears to be simple deterioration, but with many of the more powerful hyper-immune sera there is a gradual transformation to a blocking type of antibody. In some cases this is partially reversible by heat as shown with the first serum in Table VI. It will also be seen that the enhancing action of serum has become more noticeable. The second serum had kept
well until accidentally left at a higher temperature than usual. The last shows the gradual development of a pro-zone. It is of interest to note that Wiener (13) detected the formation of blocking antibodies on storage; in fact, he first noticed them in stored sera.

From many points of view the behavior of these sera is very inconvenient, but as will be shown below, it may throw light on the significance of the various forms of antibody in vivo.

**DISCUSSION**

It is of interest to compare the situation that obtains here with that occurring with Rh immunization in man. When one considers the taxonomic gulf that separates the two species, the results are surprisingly similar and might well be more so if the dosages given could be made at all comparable. In both cases it appears that a mixture of antibodies is formed, the type of result one obtains on titrating the sera depending upon their relative proportions. A pro-zone of complete inhibition is apparently much more common in mice than in man. This could be due to differences in dosage. The largest pro-zone was seen in a C57 brown animal with a large growth that had persisted for many weeks (Table III). It is difficult to believe that a situation at all comparable has ever occurred in man.

The physical conditions under which cells sensitized with partial antibodies can be made to agglutinate are undoubtedly different in the two species. Nevertheless, in both it appears that these antibodies are not homogeneous. Coombs, Mourant and Race (2) found sera which gave negative results with the blocking test and with Wiener's conglutinin test, but was positive with their antiglobulin test. Similarly, Diamond and Abelson (5) found that about 7 per cent of sera were negative both to direct agglutination and the blocking tests. The addition of the slide test enabled the presence of antibodies to be detected in nearly 100 per cent of cases as does the albumin technic (6). These authors have shown that sera showing no pro-zone and apparently weak agglutinins may have high titers of partial antibodies (6). It is not clear why some partial antibodies give a strong blocking effect and a pro-zone, whilst others do not. The precise type of titer one gets must be the resultant of the different proportions of all of them. It seems likely that the apparent drop in titer observed in the course of immunization with blood and with C1300 (see Tables I and II) is due to the presence of partial antibodies.

A further complication is introduced by the fact that we are not dealing with pure antigens. The two tumors used here contain at least two iso-antigens in common, and it is interesting to note that Dr. Snell has found that in certain crosses C1300 gives two gene ratios. It is striking that all of four A strain tumors (2 studied here and 2 in England) have appeared to contain two antigens. In the case of the growths studied here, it is possible that their proportions are different in each. Anti-sera obtained following immunization with C1300 contain an antibody reacting with C3H cells more frequently than is the case when 15091a is the antigen. This suggests that the former tumor contains more of antigen I, whilst absorption...
experiments suggest that 15091a contains more of antigen II. This quantitative difference may explain in part the difference in the antibody response shown by C57 blacks. In man, blocking antibodies are formed more frequently against antigen Rho than against the other Rh antigens.

If one attempts to generalize on the response of mice to hyper-immunization, it is possible to say only that even if the genetic constitution of the recipient is standardized as far as possible, each antigen appears to have its own peculiarities, which again may be varied according to the manner in which it is administered. If we use the same antigen, as was done with 15091a, it is apparent that the genetic constitution of the animal producing the antibody has considerable influence on the type of serum that will be obtained.

In previous publications it was pointed out that a tumor may grow in the presence of antigenic differences between transplant and host. The phenomenon of concomitant immunity made it seem likely that antibodies might be formed under these conditions (9). In the experiments reported here these were demonstrated directly in animals that were certainly going to die from the tumors. This might seem to indicate that antibodies are of minor importance. Such a conclusion is unjustified. Death from bacterial infection may occur in the presence of high titers of antibodies. Furthermore, these animals live much longer than the naturally susceptible mice which do not form iso-antibodies; over 3 months as compared with 3 to 4 weeks. It may well be that other factors than antibodies are of importance in determining the fate of transplanted growths. If the present writer has previously over-emphasised their role (10), it was in order to point out that transplantation immunity is not some novel and mysterious process, but is fundamentally the same as immunity to infection.

It has already been shown that the agglutinins can be absorbed from a serum, leaving the protective action virtually unaltered. It would appear from this that such antibodies are of much the same significance as anti-flagellar bodies in salmonella infections, etc. However, the behavior of the sera on storage suggests another explanation. Even when stored in a frozen state there is a tendency for the agglutinins to be transformed into partial antibodies, of which the blocking antibody appears to be the final product. The mouse stores its antibodies at about 37°C, and it is not unreasonable to suppose that this transformation will be greatly accelerated under these conditions. By analogy with human iso-immunization one might expect the blocking antibody to be of the greater functional significance. If this is shown to be so, we can draw an analogy between the antibody response and leukocytosis, the "complete" agglutinins corresponding to functionally immature myelocytes. Sera such as those obtained against 15091a being the analogue of an extreme shift to the left.

SUMMARY AND CONCLUSIONS

1. The A strain of mice carries at least 2 antigenic factors in its erythrocytes that are shared by the fixed tissues and are important in transplantation.

2. It has been shown that at least 3 types of iso-antibody may be produced by the mouse: (a) ordinary iso-agglutinins, (b) antibodies that need high concentrations of normal mouse serum to cause agglutination, (c) blocking antibodies, which up to the time of writing can only be recognized by their power to inhibit agglutination.

3. Sera of normal C57 blacks may contain natural iso-antibodies of the second type.

4. The proportions in which these antibodies exist in a given serum varies with the type of tissue inoculated, the interval between inoculations, the interval between inoculation and bleeding and the genetic constitution of the host. Details of the response to inoculation with whole blood and 2 tumors (C1300 and 15091a) will be found in the text.

5. In hyperimmunized animals it is common to find a distinct pro-zone with complete inhibition of agglutination up to a high dilution.

6. Animals dying as a result of tumor inoculation may have very high titers of iso-antibodies.

7. Antibodies needing serum for agglutination are found following a relatively weak stimulus.

8. Such antibodies and blocking antibodies persist longer in the circulation than do ordinary agglutinins.

9. The factor in normal plasma or serum that enhances agglutination is thermostable.

10. On storage in the frozen state there is a tendency for antibodies to be transformed towards the blocking type of antibody.

11. Ordinary agglutinin is without protective function. It is suggested that it may mature in vivo to a functional antibody. An analogy is drawn between leukocytosis and iso-antibody formation. Sera with high titers of ordinary agglutinins correspond to an extreme left shift.

ACKNOWLEDGEMENTS

It is a great pleasure to express my thanks to Dr. C. C. Little for offering me the hospitality of the Roscoe B. Jackson Memorial Laboratory. This has probably accelerated the work by about a year. Dr. Snell has been most kind in making some of his own material available.
for study, and the technical assistance of Miss Rachel Brown has been invaluable. My thanks are also due to Dr. S. Bayne-Jones for his interest and valuable criticism.

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P. A. Gorer


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