INTRODUCTION

Although induction of resistance to transplantable tumors in mice by a prior inoculation of normal mouse cells was first demonstrated many years ago, there still remain several points which require investigation. A prior publication from this laboratory (1) reviewed the literature and reported experiments which showed that the genetic interrelationships among the materials used have an important influence upon the results obtained. Various degrees of resistance were observed in heterogeneous combinations of materials, but no resistance could be induced in an inbred strain of mouse against a tumor that arose in the same strain. This supports the concept that the degree of “foreignness” of the materials introduced into the host animal is a primary factor in the reaction. However, it has never been clear what this “foreignness” actually comprised or what it was in the cell which expressed this quality.

In experiments performed to gain further information regarding the nature of the “antigen” concerned in this type of “immunity,” it was found that under the proper conditions, the power of a suspension of erythrocytes to induce immunity was comparable to that of defibrinated blood in equivalent doses (2). Thus, the plasma is apparently not concerned and, what is more important, the cell which serves as antigen does not need to have a nucleus. This seems to circumscribe and simplify the problems involved in the study of this type of immunity.

Our working hypothesis involved the idea that the antigenicity under investigation was an attribute of the cell surface and might depend upon a degree of organization above that of large molecules. With this in mind, cell suspensions were subjected to disintegration by three methods which were relatively gentle chemically and largely physical in nature—namely, osmotic laking with water, centrifugation while thawing from the frozen state, and brief exposure to high-pitched audible sound waves. It was assumed that, if the antigen were a molecular substance comparable to known antigens, it should survive one or more of these procedures and retain the power to induce a significant degree of immunity. However, the cells lost all power to induce resistance when they were broken up completely by either of the three methods of laking (2). It seemed reasonable to assume that the methods employed would produce in the cells chemical changes of only a minor degree which were not likely to be the basis for the major changes in the antigenicity observed. On the other hand, the physical changes in the cells were of a major degree. With each of the technics used, the cells were ruptured with sufficient violence to destroy almost all of the cell membranes; and, focusing upon that point, we were led to adopt, tentatively, an interpretation which was primarily physical rather than chemical.2 It then became desirable to know what results would be obtained if cells were laked by methods which would preserve most, or all, of the cell membranes. In other words, we wished to learn whether red cell stromata would by themselves induce resistance against the implantation of a tumor. The following experiments will show that they do.

MATERIALS AND METHODS

The materials and methods used were the same, except for experimental variations to be described, as those used previously (2).

2 These terms are used in the sense that the arrangement of things is physical in nature and the substance of things is chemical in nature. In using this terminology we are aware that there is no sharp line between the physical and the chemical and that ultimately this distinction may become indistinct and no longer useful. However, the distinction seems to add clarity for the present.
All the hosts were strain BALB/cN (4), approximately 8 weeks old. Both sexes were used and distributed equally throughout the experiments.

The immunizing injection was material derived from normal strain DBA/2N (4) blood in all cases and represented pooled defibrinated blood from a small number (usually six to twelve) of donors which were sometimes young adults and sometimes retired breeders, according to the availability of animals, and of both sexes. No differences ascribable to the age or sex of the blood donors were detected. The immunizing dose was always the amount of material which was derived from, or equivalent to, 0.02 cc. of defibrinated blood.

The incubation period elapsing between the immunizing injection and the inoculation of the tumor graft was always 21 days.

The transplantable tumor inoculated was DBA sarcoma 49 (2). Although this tumor originated in strain DBA (Andervont's subline of line 2), it will grow progressively in approximately 90 per cent of strain BALB/cN mice. Transplantation was performed by inoculating 0.025 cc. of a 50 per cent tumor brei subcutaneously into the right flank of the host. The mice were then observed weekly for 4 weeks; the presence or absence of a tumor was noted; and, when present, the tumors were measured for the purpose of observing growth. The data are based upon the number of tumors present 3 weeks after implantation. This time interval was selected arbitrarily and is not essential for the conclusions.

Immune control mice were given the standard dose of 0.02 cc. of washed red cells prior to inoculation of the tumor. Six groups of twenty to 30 mice each were used, one group being concurrent with each of the experimental series.

Nonimmune control mice were given nothing prior to tumor inoculation. Six groups of ten each were suitably distributed through the experiments to afford assurance that the tumor continued to grow in a high percentage of normal strain BALB/cN mice.

Red cell stromata were prepared by three methods of laking, employing saline, citrate buffer solution, and hemolytic antiserum.

A "fragility" test with graded dilutions of saline showed that 0.3 per cent saline would produce complete hemolysis in 10 minutes and leave stromata in numbers representing approximately 80 per cent of the original cells. The pH of the suspension was 7.0. Stromata prepared in this way were brought to normal tonicity by adding sufficient dry salt to the suspension at the end of 10 minutes. They were then centrifuged out, washed once with normal saline, and resuspended in normal saline. Such a suspension was injected under the dorsal skin of mice in a dose equivalent to 0.02 cc. of defibrinated blood, allowance being made for the depletion in the number of cells during treatment. Five groups of hosts, with twenty to 30 mice in a group and a total of 129, were so immunized, and 21 days later they were inoculated with the tumor.

Stromata were also prepared by means of 0.02 m sodium citrate-citric acid buffer solutions at varied hydrogen ion concentrations. After 5 minutes' exposure to such solutions, laking was complete. The stromata were centrifuged out and resuspended in normal saline. The saline suspensions were then tested for their power to induce resistance in the manner described above. Stromata prepared at pH 7.0 were used in a group of 30 animals; stromata prepared at pH 5.5 were used in two groups of 30 animals each; and a preparation made at pH 4.0 was used in a group of 30 animals.

Hemolytic antiserum was obtained by injecting a Race III male rabbit weighing 2,665 gm. with a 30 per cent suspension of washed strain DBA red cells. Doses of 1 cc. each were given intravenously on 8 successive days in each of 8 successive weeks. Two weeks after the last immunizing injection, sera from the immune rabbit and from a similar normal rabbit were harvested and prepared by the usual methods. The sera were not inactivated. When tested against a 5 per cent suspension of strain DBA red cells the antiserum (without additional complement) was hemolytic at a dilution of 1:52 and agglutinated at 1:256 after 30 minutes in a water bath at 37° C. Normal serum showed no hemolysis and weak agglutination at a dilution of 1:2. A dilution of 1:8 was selected for the experiment because, as will be described later, there was a strong contrast between the appearance of the cells in the two sera at this dilution. Red cells were suspended in normal serum and antiserum at that dilution (of serum) and incubated in the water bath for 30 minutes. Thereafter, they were tested for their power to induce resistance in the usual manner. Each preparation was tested in a group of twenty mice.

Stromata prepared with 0.3 per cent saline, as described before, were subjected to high-pitched audible sound vibrations at a frequency of 9 kc. for 90–120 seconds. Heating was prevented by circulating water at 20° C. The conditions were identical to those imposed upon intact red cells in previous work (2). In each of three experiments such vibrated stromata were injected into a group of 30 mice, and the mice were subsequently tested for...
immunity to the tumor. Another similar suspension of stromata was exposed to sound vibrations under the same conditions except that the duration was 6 minutes. These stromata were used in the usual manner in a group of 40 mice.

RESULTS

In confirmation of previously reported results, 93 per cent of untreated strain BALB/c mice had a growing transplant of the strain DBA/2N tumor at 3 weeks (six experiments totaling 60 hosts with 56 tumors); this was taken as the point of reference for results in “nonimmune” hosts. Among a total of 158 hosts “immunized” with a suspension of washed red cells in six experiments, there were 30, or 19 per cent, with tumors at 3 weeks. This also confirms previous results, with similar doses, and is taken as standard results for “immune” hosts under these conditions. Table 1 sets out these results and the various results obtained with other experimental conditions. For purposes of comparison, we have included the results of previously published experiments (2) in which the red cell suspension was laked by exposure to sound vibrations (9 kc. for 1½—2 min.) before injection into the animal.

In five experiments, stromata prepared with 0.3 per cent saline induced the same degree of resistance as would be expected with intact red cells at the same dosage. That is, there were 26 positive cases (20 per cent) in a total of 129 hosts. When examined under the phase contrast microscope these stromata were somewhat distorted but appeared to be reasonably good membranes morphologically. There was a very small number of membranes containing a few brown granules, and occasionally an intact red cell was seen. Previous investigations of the influence of dosage indicated that the small number of intact red cells present would not affect the results materially. To add strength to our opinion that the result was dependent upon the whole “equivalent” dose and not upon the few intact cells remaining, an ancillary experiment was done to see if 0.02-cc. doses of such stromata and intact red cells were additive in effect. The two doses (one of stromata and one of red cells) were given simultaneously to nineteen animals. Subsequent inoculation with the tumor yielded three positive cases (16 per cent). This is similar to a result of 15 per cent positive cases (46 hosts, seven tumors) obtained with a dose of 0.05 cc. of cells and indicates that the two doses were additive in effect.

Three experiments with stromata which had been subjected to treatment with sound vibrations similar to that previously reported for red cell suspensions yielded a result which was significantly different (P < 0.001) from that obtained with red cells. In a total of 89 hosts there were 46 successful transplants (52 per cent) at 3 weeks. This is exactly the percentage of takes previously observed after immunizing the hosts with 0.0031 cc. (equivalence in terms of defibrinated blood) of intact red cells and quite different from the 91 per cent of positive cases observed after using red cells exposed to the same sound vibrations. The difference in effect was accompanied by a difference in appearance of the two suspensions (red cells and stromata) after exposure to sound. Grossly, the red cell suspension was clear and “bright,” whereas the stromata suspension was slightly opaque and had a “silky” luster. Microscopically, the suspension of vibrated red cells contained only a rare intact cell and the small amount of debris present was of small size (1 μ or less), whereas the suspension of vibrated stromata contained a few membranes representing most, or all, of a cell and many fragments about 2—4 μ in diameter.

The suspension of stromata which had been exposed to sonic vibrations for 6 minutes was “bright” in gross appearance, like the red cell preparation, and microscopically it contained a negligible number of visible particles. Also, like the vibrated red cell suspension, it did not produce a detectable degree of resistance. In one experiment 40 “immunized” animals had 38 tumors (95 per cent) 3 weeks after inoculation. This does not differ from “nonimmune” controls.

Stromata prepared by means of 0.02 M citrate buffer induced a degree of resistance which varied

<table>
<thead>
<tr>
<th>Antigen injected</th>
<th>Total mice</th>
<th>Total tumors</th>
<th>Percent positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (nonimmune controls)</td>
<td>60</td>
<td>60</td>
<td>93</td>
</tr>
<tr>
<td>Washed red cells (immune controls)</td>
<td>158</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>Washed red cells (after 1½—2 min. sonic vibration—from [2])</td>
<td>55</td>
<td>50</td>
<td>91</td>
</tr>
<tr>
<td>Stromata prepared with 0.3 per cent saline, pH 7.0</td>
<td>129</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>same—after 1½—2 min. sonic vibration</td>
<td>89</td>
<td>46</td>
<td>52</td>
</tr>
<tr>
<td>Stromata prepared with 0.02 M citrate buffer, pH 7.0</td>
<td>30</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>same—after 6 min. sonic vibration</td>
<td>40</td>
<td>38</td>
<td>95</td>
</tr>
<tr>
<td>Stromata in rabbit antiserum</td>
<td>20</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>Cells in normal rabbit serum</td>
<td>20</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

See text for statistical analyses.

* All doses were equivalent to 0.00 cc. of blood. Incubation period 91 days. Tumors represent positive cases 5 weeks after tumor inoculation.
with the pH of the buffer used. Thirty animals immunized with stromata prepared at pH 7.0 had four tumors, or 13 per cent. That result is not significantly different from those obtained with either intact red cells or stromata prepared with saline. However, stromata prepared at pH 5.5 were less effective and yielded an intermediate result. Sixty animals were immunized with such stromata and, of these, twenty, or 33 per cent, were susceptible to the tumor. That result differs significantly from both nonimmune controls (P < 0.001) and immune controls (P = 0.09). It is also different from results obtained with stromata prepared with saline (P = 0.05) and stromata prepared with citrate buffer at pH 7.0 (P = 0.04). Stromata prepared at pH 4.0 were even less effective as an antigen. Of 30 animals immunized with such stromata, 28 (93 per cent) were susceptible to the tumor. Although this result is different (P < 0.01) from that in nonimmune controls, it also differs significantly from all other groups of immune animals. We did not observe any difference in the microscopic appearance of wet mounts of these three preparations of stromata which correlated with the results, but the stromata prepared at pH 4.0 were unique in one respect; the brown granules which can be seen both inside and outside the membranes in such preparations were immobile after exposure to pH 4.0, whereas in all other preparations (including that with saline) they were dancing. Otherwise, these stromata all had the same approximately normal, though somewhat distorted, appearance as those prepared with saline.

Stromata prepared by means of hemolytic antiserum produced a result which was the same as that obtained with stromata prepared with saline and was not significantly different from that following the use of citrate buffer solution at pH 7.0. Of twenty hosts in this experiment, four, or 20 per cent, were susceptible to the tumor. Among the twenty additional control animals which were immunized with unlaked cells in normal rabbit serum there was one (5 per cent) positive case. These two results do not differ significantly (P = 0.16) between themselves or from immune controls (P = 0.093 and 0.13, respectively), but they are both different from nonimmune controls (P < 0.001). However, there was a difference in the appearance of the cells after treatment with the two sera which is of some interest. The suspension containing antisera showed complete hemolysis and strong agglutination, while that containing normal serum showed no hemolysis and minimal agglutination.

Under the phase contrast microscope wet preparations of these cells showed normal discoid appearance, "flicker" (3), very little agglutination, and only an occasional ghost in the normal serum, whereas in antiserum the cells were spherical, clumped, without "flicker," and only an occasional cell contained hemoglobin.

**DISCUSSION AND CONCLUSIONS**

A discussion touching upon all the work of others which might have some bearing upon these experiments would be unduly long. Discussions of such work can be found in reviews by Woglom (16), Spencer (15), and Snell et al. (13). Experiments with animals of unknown or heterogeneous constitution will not be mentioned, because they involve such a large complex of variables, many of which are unknown or uncontrollable, that we are unable to relate them to this work with certainty.

That special part of the field of tumor immunity which comprises the alteration of the susceptibility of animals to tumor implantation by pretreatment with nonliving materials has been reviewed by Snell et al. (13). Greatest interest attaches to the effect of the injection of lyophilized tissues, which has been studied by Snell, Kaliss, and their co-workers (10, 11, 13, 14). They have usually observed an enhancement of susceptibility to tumor implantation, but in some cases inhibition was observed, and the latter effect raises a question of the relationship of their phenomena to ours. However, at present, the differences between their experiments and ours seem more impressive than the similarities, for the following reasons. Firstly, the observations which they have reported include a qualitative change in the effect, i.e., enhancement in most cases and inhibition in some, whereas we have observed only inhibition or no effect, according to conditions. With variation of strains of mice in heterogeneous systems they obtained the qualitative reversal of effect, as well as the expected quantitative variations, whereas with variation of strains we obtain only quantitative variations of the same qualitative effect. Secondly, with variation of dosage they have observed a qualitative change in the effect, whereas we have seen only a quantitative effect of dosage. It must be remarked that their dose was expressed in terms of dry weight, while ours was expressed in volume. However, rough calculations based upon specific gravity and water content indicate that the seven dosage levels employed by us (i.e., 0.006–0.2 cc. equivalence of cells) and two higher levels employed by earlier workers (15) (0.5 and 0.5 cc. of defibrinated blood) would, if expressed in dry weight, range from well above the higher doses with which they obtained enhancement down to the lower doses with which they obtained inhibition; we always observed inhibition. Thirdly, their effect was ex-
pressed (14) as a difference in the size of the tumors which, in the case of inhibition, might be accompanied by an increase, decrease, or no change in the percentage of hosts susceptible to the tumor, whereas our effect was always seen as a decrease in the percentage of hosts susceptible to the tumor. Lastly, but in our opinion not least, their effect was observed after freezing, drying, and powdering the tissues, whereas the effectiveness of our “antigen” was destroyed by disintegration of the cell, and the disintegrated material gave rise to no enhancing effect detectable by our methods. One must conclude that the material with which they worked cannot at present be compared to the antigen which we are investigating. Furthermore, without prejudice to their data, we are of the opinion that thus far it is a little easier to associate their work with studies on “hypersusceptibility”—to borrow a term from the early literature (16)—especially since this is the effect more often observed, and to postpone a decision regarding its relationship to “induced resistance” with which this paper is concerned.

Regarding the induced resistance or immunity to transplantable tumors which can be produced by pretreatment with cells, Woglom (16) and Spencer (15) adopted the view (prior to the publication of [13]) that there was no satisfactory evidence that resistance could be induced by nonliving materials. The investigation most often referred to in this regard is that of Haaland (8), who said that cells “devitalized” by freezing and grinding were devoid of power to immunize against transplantable tumors. It now appears that such a method of killing cells would not only “devitalize” them but would also “disorganize” them, and the latter may be as important as the former. Formerly we accepted, tentatively, the then existing evidence that the power to induce resistance was a property of “intact, living cells.” With later work (2), the emphasis shifted to the word “intact,” and we began to suspect that architecture or organization (above the molecular level) might be an important determining factor. The results reported here seem to give encouragement for maintaining such a viewpoint on a working basis until more of the subtleties are known.

The data of Table 1 leave no doubt that a high degree of resistance against the implantation of a tumor can be induced by a prior injection of red cell stromata. Furthermore, the effectiveness of the stromata in inducing such resistance does not appear to be less than that of an equivalent dose of red cells. The aggregated data from experiments in which stromata were prepared at pH 7.0 show 34 tumors in 179 hosts, or 19 per cent positive; this is the same result as was obtained in 158 hosts immunized with washed red cells. Generalization beyond the bounds of these experiments may not be justifiable, but there is no apparent reason for believing that these results will turn out to be unique and limited to the conditions of these experiments.

The principal effect of this observation is to contradict the notion that immunity to the implantation of tumors can be induced only by “living” cells (possible exceptions are discussed in [13]). Until some future date when the boundary between “living” and “dead” can be accurately defined—if it ever can be—it would be fruitless to argue this point at length. Nevertheless, it is the opinion of the authors that it would be going too far to call these stromata “living” in the ordinary meaning of that term and that this point has lost whatever significance it might have once had.

Whatever the nature of the antigen involved in this type of immunity is, the fact that it is not limited to the intact living cell seems to open new possibilities for further elucidation. Thus far we have found that, of six methods of laking reported, those technics which left few large fragments of the cell membrane ablated the power to induce resistance, whereas those technics which left many large fragments of the cell membrane did not. Furthermore, when treated with sound waves red cells quickly lose all their antigenic power (of this type), and this is accompanied by reduction to small particle size, whereas stromata similarly exposed are not completely broken up and do not lose all antigenic power. Only after exposure to sound waves for 3 or 4 times as long a period were stromata observed to be reduced to small particle size and to become nonantigenic. It is this apparent correlation between the degree of disintegration of the material and the degree of destruction of antigenicity which leads us to adopt tentatively a physical viewpoint of the phenomena, although we recognize that other viewpoints are tenable.

Undoubtedly some minor chemical alterations at the molecular level of organization take place in some or all of the procedures, but it is difficult to believe that such form the basis for the observations. This viewpoint with respect to the effect of high-pitched audible sound is supported by the work of others. In immunologic studies of mammalian cells Henle, Henle, and Chambers (9) reported that sound vibrations at a frequency of 8.9 kc. did not

4 A possible explanation of the difference in resistance to sound vibrations may lie in the way that energy would be released at the interface between materials of different density. In the case of stromata, saline is both inside and outside the membrane, whereas cytoplasm (etc.) is inside the intact cell membrane and saline is outside.
destroy the head specific, tail specific, or species specific antigens of spermatozoa of man, dog, rabbit, guinea pig, or bull. The time of exposure in their work varied from 7 minutes to 1 hour, and the temperature was "not higher than 20° C." The morphology of the head or tail was not destroyed by these treatments except to separate the head and tail, and this, according to their interpretation, exposed a fourth antigen which was brought to the surface at the break. This is in contrast to our observations but supporting rather than contradicting, because in their work more rigorous exposure to sound vibrations did not destroy the cells nor the antigens. With respect to bacterial antigens, Chambers and Flosdorf (5) reported that the Vi antigen of Eberthella typhi survived vibration at a similar frequency for 45 minutes at 15° C. Mudd et al. (12) noted that under the same conditions the antigen responsible for the phagocytosis of a strain of Streptococcus hemolyticus survived for 1 hour. Chambers and Weil (6) reported that it required 60—75 minutes of such vibration to render the "irritating substance" of pneumococci nonantigenic. Crystalline egg albumin was not distinguishably different immunologically from native protein as stated, only approximately intact representatives of the cell surface. Contrariwise, the best appearing stromata with which we have worked had been exposed to 0.05 per cent formaldehyde for 15 minutes (unpublished experiments), and these were devoid of the power to induce resistance (twenty hosts, twenty tumors). Similarly, heating at 56° for 30 minutes produced stromata which had an appearance similar to potent ones (unpublished experiments) but they also were devoid of immunizing power (twenty hosts, twenty tumors). The deleterious effect of lowered pH was not accompanied by commensurate morphologic changes. Thus, deleterious chemical effects may or may not be accompanied by correlated morphologic effects. A similar (but obverse) point can be made regarding the cells treated with serum; here, there were marked differences in appearance of the cells in the two sera but no difference in their immunizing power.

The existing data suggest that a degree of organizational integrity is essential in the nature of the "antigen" under investigation, but this does not demand either a whole cell or an undistorted one. In addition, as would be expected, relatively gentle chemical changes can also effect the potency of the antigen, but some of these may not destroy the potency if sufficient organization remains. One may imagine that in part, at least, the "foreignness" of the antigen may depend upon not only what is present but also how it is arranged. After all, this may be only a restatement of the basic concept of stereochemistry with reference to a different level of biological organization, and the same difference may be all that distinguishes this concept from the well known concepts of Landsteiner and Pauling.

An alternative interpretation might be that the essential nature of the antigen does not depend upon a supramolecular aggregate but that the development of immunity after injection of the antigen depends upon such an aggregate. Thus, the finely divided antigen might be functionally ineffective because of increased lability, quick removal from the depot, or for some other reason. A firm choice between these alternatives can be made only after further experimentation. The fact remains that no immunity was observed following the injection of finely divided antigen.

SUMMARY

Previous observations that a prior inoculation of washed red cells would, under proper conditions, induce a high degree of resistance against a subsequent inoculation of tumor were confirmed.

It was further observed that red cell stromata were capable of inducing resistance which was not distinguishably different in degree from that induced by intact red cells. This result was attained with stromata prepared by means of 0.3 per cent saline, 0.02 M citrate buffer solution, or hemolytic rabbit antiserum at or near neutrality. However, lowering the hydrogen ion concentration of the laking fluid to pH 5.5 or pH 4.0 produced a corresponding lowering of the degree of induced resistance.

Exposure of stromata to high-pitched audible sound vibrations ablated their power to induce resistance, but it was necessary to expose stromata
for longer periods of time than was required to produce the same effect in red cells. Under the influence of sound vibrations, the degree of loss of antigenicity of red cells and stromata was correlated with the degree of morphologic disintegration.

There was no discernible morphologic change which could be correlated with the graded effect on antigenicity of lowering the pH. On the other hand, there was no correlated change in the antigenicity accompanying the morphologic changes produced by antiserum.

It is concluded that the antigen involved in induced resistance is not limited in occurrence to the intact living cell. This antigen is sensitive to chemical change but, more important for future work, it seems to depend upon some degree of organization (or possibly aggregation). The level of organization required appears at present to be somewhere above the molecular level but not so high as that of the cell. Tentatively, one may suspect that the arrangement of its constituent parts is critical in the nature of the antigen, and this has been adopted as a working hypothesis.

REFERENCES

Resistance to Tumor Implantation Induced by Red Cell Stromata

Morris K. Barrett and Walter H. Hansen