

The Antibody Response to Tumor Inoculation

Improved Methods of Antibody Detection

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The literature on the immunological aspects of tumor transplantation has been well reviewed by Hauschka (9), so it is necessary only to point out certain technical problems that form the basis for the present communication.

The most striking recent advances in our knowledge of the genetics of tumor transplantation have been due to the work of Snell and his colleagues on the H-2 locus (13) and the identification of two further histocompatibility loci (12). The H-2 locus received its title from an A strain antigen called antigen II by the senior author (3, 4), which Gorer, Lyman, and Snell (7) showed to be linked to the locus for "fused" (an anomaly of the tail). This antigen (more correctly "antigens," see Table 5) was recognized by erythrocytic agglutination and shown by absorption experiments to be present on the fixed tissues as well. More recent progress by Snell and his colleagues has been purely genetic, the results of tumor transplantation being taken as an index of antigenic compatibility. This has shown that the H-2 antigens play a dominating role in the transplantation of all neoplasms and are therefore of considerable interest to the oncologist. Valuable though these studies are, the methods used have certain inherent drawbacks (4, 7), and additional serological data are necessary.

However, a serological study of the histocompatibility antigens has been handicapped by two technical shortcomings: In ordinary saline media strong agglutination could be obtained with regularity only with A strain red cells; other strains, such as C3H sometimes gave good titers, but the results were unpredictable—while with C57 blacks only one success occurred in many trials. Furthermore, the behavior of sera on storage was most irregular even when the sera were kept in the frozen state. Some sera of good titer were apparently inactive after 18 hours, while others might keep for weeks. Evidence was obtained that the antibodies were not really destroyed on storage

but were transformed into "incomplete" antibodies (5). Later it was found that such antibodies might agglutinate erythrocytes if they were suspended in suitably absorbed human sera (6). Unfortunately, it was subsequently found that this was unreliable, and the present communication deals with the development of a much more dependable technic.

MATERIALS AND METHODS

The mice used have been those of strains A, C57BL, C3H, and Balb/C. The first two of these were maintained by the senior author from 1934 till 1940, at which time some breeding stock were given to the Imperial Cancer Research Fund. Our present stocks are descendants of these and were kindly supplied by Dr. James Craigie, from whom the C3H mice were also obtained. The Balb/C mice were left here in 1946 and were said to have come from the National Cancer Institute, Bethesda, Md.

The four strains are capable of supplying twelve antigen-antibody systems, all of which have been tested with either normal or malignant tissues as antigens (2).

In developing a suitable technic we concentrated on the cells of C57BL mice, since there was good reason to suppose that a technic successful with them would be of wide applicability.

The antigenic stimulus most commonly used was the C57 lymphoblastic leukemia E.L. 4 (6). Following the subcutaneous inoculation of about 100,000 cells into mice of the other three strains, a mass appeared in about 5 days, regression began about the 10th day and was usually complete by about 14 days (regression occasionally failed to occur). The antibody response to primary inoculation was poor with this tumor. One or two further inoculations of from 50–80 million cells were given by the intraperitoneal route. The spacing of the other inoculations was somewhat irregular—the second inoculation was generally given 3–4 weeks after the first, and the third 10–14 days after the second, the mice being bled 10 days after the last inoculation. Good titers were often obtained after two inoculations, but we recommend three, particularly with A anti-E.L. 4.

The sera from about six mice were pooled and either lyophilized in ampules containing 0.1 ml. or stored without drying in similar amounts at -20°C . In either case it is most important that the ampules be well sealed. Repeated freezing and thawing appeared deleterious with some sera; this contra-indicated storage in bulk. The lyophilized sera were also kept at -20°C ., although this is probably unnecessary.

The methods of bleeding mice and absorbing human sera were those used previously (5, 6), but

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the technic of reading the results has been modified to conform with that often used in Rhesus typing in this country. Equal volumes (usually 0.025 ml.) of cell suspension and antibody were incubated in round-bottomed precipitin tubes at 37° C. for 1 hour or more, by which time all the cells should have settled. The tip of a Pasteur pipette was then placed directly on the cellular deposit, part or all of which was then withdrawn, together with some supernatant fluid, without any attempt to mix the two. The cells and supernatant were then streaked vertically on a slide. The contents of up to five tubes may be put on the same slide (fewer are preferable at first), which is then gently rocked. The utmost delicacy is essential in all these manipulations. Weakly agglutinated cells tend to separate on a slide after rocking has ceased, and there should be no undue delay in reading the slides microscopically where this is necessary. Attempts to save time by transferring

clump and broke up but little on the slide. As agglutination became weaker the deposit remained solid in the pipette, but broke up to an increasing degree on the slide. Finally a macroscopic diagnosis became impossible, although definite agglutination could be seen microscopically.

Every sample of serum has been tested against the red cells of all four strains as a check on the specificity of the reactions. Of 110 samples tested in this way, one agglutinated the cells of mice from all strains including the A strain which supplied the serum. No explanation of this is available. It is definitely not a common occurrence.

RESULTS

When working with normal human serum, it appeared that the best results were obtained with sera from people with mild indispositions, whose erythrocyte sedimentation rate (E.S.R.) was raised. We therefore obtained sera from patients

TABLE 1
THE DEMONSTRATION OF AGGLUTINATION BY MYELOMA SERUM AND VARIOUS MUCOID SUBSTANCES
Antibodies: C5H anti-EL. 4
Cells: C57 blacks

CELLS IN	AGGLUTINATION OF RED CELLS BY ANTIBODY DILUTED ONE IN								
	CONTROL	4	8	16	32	64	128	256	512
N.H.S.*+4 per cent globulin	—	—	—	—	—	—	—	—	—
Myeloma serum case 3	—	+++	+++	+++	+++	++	++	±	tr†
N.H.S.+cord (20 per cent)‡	-r§	+++	+++	+++	++	±	tr.	—	—
N.H.S.+cyst fluid (1:5)	-r	+++	ac#	ac	+++	++	++	+	±

* N.H.S. = normal human serum.

† tr = trace of agglutination.

‡ The muco-protein was obtained by extracting 1 gm. of minced and ground umbilical cord and 5 ml. of normal saline at 37° C. for 1 hour.

§ -r = slight rouleaux formation.

r = marked rouleaux formation.

ac = almost complete agglutination.

too many tubes to slides have given rise to serious error.

Duplicate controls without antibody were set up routinely. The deposit from these appeared somewhat granular when drawn up into the pipette. The granules broke up rapidly on rocking. If examined under the microscope before this had occurred, it was generally noted that these granules had quite a different appearance from those seen in true agglutination. The former were roughly spherical, and single cells broke away very readily; the latter appeared flat and sticky. While this distinction is difficult to describe, it becomes clear with but little practice. A more formidable difficulty was the formation of rouleaux. In this case the cells adhered by their concave surfaces to form short strings, whereas agglutinated cells adhered at random. If of mild degree, this was unimportant, but sometimes the strings stuck together in a manner that mimicked true agglutination (it is called pseudo-agglutination in human blood grouping).

Strongly agglutinated cells came away as a solid

suffering from a wide variety of diseases, all of whom had an E.S.R. of over 70 mm. in the 1st hour. Such sera gave better enhancement than those obtained from healthy donors, but complete failures were still frequent. Addition of human or bovine γ -globulin to normal human serum gave much the same result as the bulk of pathological sera. However, among the latter, outstanding and consistent success was obtained with sera from certain patients with myelomatosis. Six such sera were examined; three of these contained no abnormal serum protein component and had no exceptional powers of enhancing agglutination, while the other three showed different abnormalities; but all gave excellent agglutination with antibodies which were apparently inactive when tested in other ways.

Shapiro, Ross, and Moore (11) purified an abnormal component and found that it contained a considerable amount of carbohydrate. We therefore tested some naturally occurring muco-proteins and muco-polysaccharides. The first to be examined were an extract of human umbilical

cord and the contents of a human pseudo-mucinous ovarian cyst. The results are shown in Table 1. It will be seen that the pseudo-mucin gave a titer comparing favorably with that of the myeloma serum, while normal human serum plus globulin was ineffective. The extract of umbilical cord seemed somewhat less efficacious than the pseudo-mucin. The concentrations to be used had to be determined empirically; both caused very marked rouleaux formation at strengths much above those shown in the table, while the effectiveness fell off rapidly on further dilution.

Crude hogs' gastric mucin gave results comparable with those given by pseudo-mucin, while some purified "blood group" polysaccharides gave very strong rouleaux formation with comparatively feeble enhancement, as shown in Table 2.

We have examined a large number of dextran preparations with mean molecular weights between 30,000 and 400,000. Those in which this value was above 100,000 gave very marked rouleaux formation even at very low concentration. The ordinary commercial preparation made up in saline also suffered from this defect. However a preparation known as "Intradex Salt Free," marketed by Messrs. Glaxo Ltd., Greenford, Middlesex, gave excellent results. This is made up as 10 per cent solution of dextran (molecular weight 100,000) in 5 per cent glucose.

A comparison of decreasing concentrations of dextran and 1:5 cyst fluid is shown in Table 3. The dextran was brought to the requisite strength by dilution in saline. Further experience has shown that at the 3 per cent level one may have trouble

TABLE 2

A COMPARISON OF THE ENHANCING EFFECTS OF OVARIAN CYST FLUID (HUMAN) AND VARIOUS FRACTIONS OF HOGS' GASTRIC MUCIN

Antibodies: A anti-E.L. 4
Cells: (Balb/C × C57BL)F₁

CELLS IN	AGGLUTINATION OF RED CELLS BY ANTIBODY DILUTED ONE IN								
	CONTROL	2	4	8	16	32	64	128	
N.H.S.* +1:5 cyst fluid	—	+++	+++	++±	+++	++	±	±	?
N.H.S.+4 per cent crude mucin	—r†	+++	+++	+++	++±	+	±	±	tr‡
N.H.S.+2 per cent crude mucin	—r	++±	++±	++	+	tr	tr	tr	—
N.H.S.+2 per cent purified polysaccharide (High "A" activity)	r	+++	+++	++±	+	tr	r	r	
N.H.S.+purified polysaccharide (High "H" activity)	r	+++	+++	++±	+	tr	r	r	

* N.H.S. = normal human serum.
† —r = slight rouleaux formation.
r = marked rouleaux formation.
‡ tr = trace.

The pseudo-mucin was so convenient to use that other naturally occurring substances were not studied in any great detail. In the first experiments the pseudo-mucin was dissolved in the human serum, but subsequently it was found more convenient and slightly more effective to use it as a vehicle for antibody dilution and normal human serum for suspending the cells. Thirteen different antisera were used with this system and gave satisfactory results with cells of C57 blacks and various hybrids derived from them, including a back-cross to the A strain.

Pseudo-mucinous cysts are not rare, but they differ very widely in the viscosity of their contents; it therefore seemed desirable to try to obtain some more standardized material. Grubb (8) had found that ordinary commercial dextran would give agglutination of incomplete Rh antibodies as well as enhance certain antibacterial sera. Ikin (quoted by Mourant [10]) has obtained good results with a number of dextran preparations of high molecular weights.

with controls. At the 2 per cent level the titer with the antiserum shown in the table was better than that shown by 1:5 cyst fluid. This was not shown in all cases, and it is possible that some modifications in technic might render them equally effective; but the fact that the dextran preparation can be reproduced indefinitely makes it preferable.

It would be advantageous if one could dispense with the necessity to absorb human serum. Both cyst fluid and dextran have been examined with the red cells suspended in decreasing dilutions of human serum. The results were essentially similar in either case, an experiment where dextran was used being shown in Table 4. The chief effect shown is an increasing pro-zone as the amount of human serum was decreased. Some antisera showed pro-zones even in the presence of undiluted human serum, and such zones may be very marked in genetical experiments; we therefore generally employed undiluted human serum for genetical experiments and used a one in two dilution in the routine testing of antisera.

We would emphasize that no dextran preparation should be accepted as satisfactory unless C57BL cells show good agglutination. We recently received a sample from America which had given good titers with many strains other than C57BL at the Roscoe B. Jackson Memorial Laboratory. In our hands, it gave fairly good agglutination of strain A cells and strain C3H cells, but very feeble agglutination of C57 cells, while with the same antiserum and our own dextran the titer with the blacks was 1024. The use of such a preparation can give very misleading results.

DISCUSSION

In addition to the technics of erythrocytic agglutination that have been described above, Amos

(1) has devised a technic for demonstrating iso-antibodies with leukocytes. The application of these methods to various problems will be described in future communications. However, it is interesting to note that the H-2 antigens "D" and "K" have been identified serologically, as have two other H-2 antigens in C57 blacks. In addition to these, a large number of antigens are as yet unclassified, some of which do not belong to the H-2 system.

Leukocytic agglutination has enabled us to detect antibodies when erythrocytic agglutination has failed. Unfortunately the leukocytes have to be obtained from the spleen or peritoneal exudates; this limits its usefulness in genetic studies. The leukocytes of our four strains do not differ

TABLE 3
A COMPARISON OF THE EFFECTS OF OVARIAN CYST FLUID AND DECREASING AMOUNTS OF DEXTRAN ("INTRADEX SALT FREE")*

Antibody: Balb/C anti-E.L. 4
Cells: (Balb/C × C57BL)_F₁, suspended in N.H.S.

ANTIBODY DILUTED IN	AGGLUTINATION OF RED CELLS BY ANTIBODY DILUTED ONE IN										
	CONTROL	2	4	8	16	32	64	128	256	512	1024
1:5 cyst fluid	-	-	++	+++	+++	++	±	tr	-	-	-
4 Per cent dextran	?	+++±	+++	+++	+++	+++	+++	+++	+++	+++	+++
3 Per cent dextran	-r	+++±	+++	+++	+++	+++	+++	+++	+++	+++	+++
2 Per cent dextran	-	tr	+++	+++	+++	+++	+++	+++	+++	+++	?
1 Per cent dextran	-	-	+++±	+++	+	tr	-	-	-	-	-

* The dextran was diluted in saline. For abbreviations, see footnotes to Table 2.

TABLE 4
THE EFFECT OF DEXTRAN AS A VEHICLE FOR ANTIBODY DILUTION WITH CELLS SUSPENDED IN DECREASING AMOUNTS OF HUMAN SERUM*

Antibody: C3H anti-E.L. 4
Cells: (Balb/C × C57BL)_F₁

ANTIBODY DILUTED IN	CELLS SUSPENDED IN	AGGLUTINATION OF RED CELLS BY ANTIBODY DILUTED ONE IN										
		CONTROL	4	8	16	32	64	128	256	512	1,024	2,048
Saline	N.H.S. 1:1	-	++	tr	-	-	-	-	-	-	-	-
Saline	N.H.S. 1:40	-	-	-	-	-	-	-	-	-	-	-
3 Per cent dextran	N.H.S. 1:1	-r	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++±
3 Per cent dextran	N.H.S. 1:4	-	++	+++	+++	+++	+++	+++	+++	+++	+++	+++±
3 Per cent dextran	N.H.S. 0:16	-	-	+	+++	+++	+++	+++	+++	+++	+++	+++±
3 Per cent dextran	N.H.S. 1:40	-	-	-	f+++	f+++±	f+++	f+++	f+++	f+++	++	+

* For abbreviations, see footnotes to Table 2.

† f = The red cells are spread in a film on the bottom of the tube, in which case they may be firmly stuck to the glass and difficult to draw out without using enough force to break up the clump.

TABLE 5
A COMPARISON OF THE SENSITIVITIES OF RED CELLS OF STRAINS A, BALB/C AND C3H IN THE HUMAN SERUM-DEXTRAN SYSTEM

Antibody: C3H anti-Balb/C
Antigen: H-2 D

CELLS OF STRAIN	ANTIBODY DILUTED ONE IN						
	128	256	512	1,024	2,048	4,096	8,192
A	+++	+++	+++	+++	+++	++	++
Balb/C	+++	+++±	+++±	++	+	-	-

Antibody: (Balb/C × C57BL)_F₁, anti-A
Antigen: H-2 K

CELLS OF STRAIN	ANTIBODY DILUTED ONE IN						
	128	256	512	1,024	2,048	4,096	8,192
A	a.c.	+++	+++	+++	+++	+++±	++
C3H	+++	+++	+++±	+++±	+	-	-

appreciably in sensitivity to iso-agglutination, whereas red cells do so very markedly. Those of the A strain are by far the most sensitive. Table 5 shows a comparison of A and Balb/C mice with respect to anti-D, and A and C3H mice with respect to anti-K, the A strain giving higher titers in each case. This superior sensitivity of the A cells is not equally striking with all sera but is of great importance when studying the appearance and disappearance of antibodies from the circulation. Thus, if a small dose of Balb/C blood is given to C57BL mice (to give anti-D) only A strain cells may react. The erythrocytes of C3H mice seem of about the same sensitivity as those of Balb/C,

while those of C57BL mice are the least sensitive and thus form the most critical test of technical adequacy.

As a producer of iso-antibodies the A strain offers the greatest difficulties. Only one sample of A anti-C3H has been tested. This gave no erythrocytic agglutination and feeble leukocytic agglutination. Five samples of A anti-Balb/C have been tested. One of these gave feeble agglutination of Balb/C red cells with marked zoning; leukocytic agglutination was observed with all of them. Sixteen samples of A anti-C57BL have been tested, one against a mammary tumor and fifteen against E.L. 4. The former gave a poor titer of red agglutinins while two of the latter were inactive. The best titer we have obtained is 4096, while most lie between 256 and 512. As a rule the antisera keep very well in the frozen state at -20°C ., but one sample deteriorated unexpectedly after a few weeks; none of our lyophilized samples have yet been found to do so.

Sera from the other three strains gave rise to very few disappointments. A sample of C3H E.L. 4 showed no drop in titer after being kept frozen for 8 months, while a lyophilized sample of Balb/C anti-E.L.4 showed full activity after 4 weeks at room temperature. Sometimes we have found that some sera, apparently inactive when fresh, have given agglutination after storage of several days in the frozen state. It was rare for pro-zones to develop in frozen or lyophilized sera, but this has been observed in both cases.

The fact that marked zoning may occur in the human serum-dextran system makes it seem likely that some antibodies will be detectable only by means of an antiglobulin test, as is known to occur with human iso-antibodies. A satisfactory test of this type appears very difficult to develop in mice. Some positive results have been obtained, but the test is not yet sufficiently sensitive to be of practical use.

SUMMARY

Up to the present the most efficient method of studying iso-agglutinins in mice has been to suspend the erythrocytes in normal human serum, but experience has demonstrated this technic to be unreliable, particularly with the cells of C57BL mice. The enhancing power of a number of pathological sera was therefore tested; the sera from a number of cases of myelomatosis gave exceptionally good enhancement. This could be reduplicated by suspending the red cells in normal human serum and adding certain naturally occurring mucoid substances to the saline used as a vehicle for the dilution of antibody. Of these, pseudo-

mucin from a human ovarian cyst was found to be most efficient.

Several preparations of dextran were tested, most of which gave intense rouleaux formation of mouse cells. One ("Intradex Salt Free") gave excellent results and is now in routine use as a vehicle for antibody dilution.

We feel that the ability to obtain good agglutination of the cells of C57BL mice is an essential test of the efficiency of any system.

Technical failures have been found to be due to the following causes: (a) errors in the technic of reading, such as rough handling of the cells; (b) unsuitable preparations of dextran; and (c) inadequate immunization.

Certain antisera are inactive in the human serum-dextran system, particularly those produced in the A strain. It is probable that an antiglobulin test is essential in such cases.

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