THE "INCOMPLETE" ANTIBODY FORMATION IN WEBSTER MICE

by

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INTRODUCTION

History

The concept of immunity originated from the knowledge that after recovery from an infectious disease a person usually would not have another attack of that particular disease. The first application of this idea was with Jenner's vaccination against Smallpox using live virus from naturally acquired Cowpox. The search for the explanation of this and related phenomena led to the discovery of a peculiar sort of substance in the blood serum, which was named antibody. This substance was formed not only as a result of infection, but also after the administration of certain complex poisons such as toxins, and foreign proteins. Research was also done using dead bacteria. It was not long before several workers, namely; Bordet, Tschistositch, Delfanti, Carbone, von Dungern, Landsteiner and Uhlenhuth, found that immunization against microbes, toxins and proteins is only a particular instance of a general principle, i.e., a foreign substance can induce the formation of a specific antibody, which combines with that substance in some measurable way.(78)

Definition of an Antigen

A substance which, when injected into the tissues of an

animal, causes the appearance, after a suitable length of time, of antibodies in the cells, blood serum or other body fluids. Usually the reaction is specific in that each antigen stimulates the formation of antibody for itself and no other antigen even though it may be closely related; and the reaction takes place only when the antigen is foreign to the internal environment of the animal into which it is injected.(9)(13)(17)(24)(36)(113)

General Properties of Antigens

Antigens are high molecular weight substances usually protein in nature, which possess many immunologically "active" chemical groups. They induce antibody formation when injected into an animal. The specificity of antigens depends upon their chemical composition. An antigen's specificity may be altered by introducing various chemical groups such as carboxy or phenolic or by certain modifiers such as acids and bases. Neutral substances have been found to be less effective. It is the present belief that the arrangement of the amino acids in the protein chain, particularly the terminal amino acid, determines the serologic character of the protein.

Antigens which do not cause the formation of antibodies after their injection into an animal but which do react with antibodies in a test tube are termed haptenes. At the present time there appear to be two kinds: 1) complex haptenes, which combine visibly with antibody; 2) simple haptenes, which combine invisibly and prevent visible reactions. The most intensively studied haptenes

have been the polysaccharides, lipids and simple chemical substances, e.g., amino acids. Pure polysaccharides can on occasion act as complete antigens; lipids when combined with proteins may also become antigenic.(9)(13)(17)(24)(36)(113)

Definition of an Antibody

A protein, which is formed by the animal in response to the presence of antigen in the tissues and which combines specifically with the antigen.(9)(13)(17)(24)(36)(113)

Types of Antibodies

1.) Agglutinins - Antibodies formed in response to the injection of bacterial or other cells. When mixed with the homologous cells the antibody will immobilize them if they are motile, then aggregate, or agglutinate the cells with the formation of clumps.

2.) Autoagglutinins - Globulins, believed to be antibodies, which agglutinate a person's own red cells, white cells or platelets. One variety usually reacts best at low temperatures and is therefore called a "cold agglutinin". The role of autoagglutinins in the production of disease has not been clearly defined.

3.) Isoagglutinins - Antibodies which occur naturally or which can be induced within a single animal species.

4.) Antitoxins - Antibodies formed in response to the injection of toxins. When mixed with toxin the antibody specifically neutralizes its posionous qualities.

5.) Viral neutralizing - Antibodies which when mixed and incubated with an infectious agent, render it non-infective.

6.) Ablastins - Antibodies which prevent the multiplication of the invading micro-organism.

7.) Opsonins - Antibodies which render bacteria susceptible to ingestion by phagocytes.

8.) Lysins - Antibodies which bring about a dissolution or lysis of bacterial or other cells in the presence of complement.

9.) Precipitins - Antibodies which aggregate soluble antigens.

10.) Heterophile (Forssman Group) - Antibodies produced in response to the injection of a wide variety of tissues and causing the lysis of sheep red blood cells in the presence of complement.

11.) Reagins - Antibodies which are demonstrated in the serum of spontaneously allergic (atopic) individuals. They have particular affinity for human skin and appear to be responsible for cutaneous wheeling reactions.

12.) Blocking - Antibodies formed by the repeated injection of minute amounts of allergens into allergic patients. The antibody specifically "neutralizes" the allergen so that the mixture does not produce a skin reaction in sensitive subjects.

13.) Incomplete (Univalent) - Antibodies which, when mixed with antigen, will coat the antigen but will not cause a visible reaction in saline.

14.) Properdin - A normal constituent of serum which combines

weakly with foreign antigenic substances, helps destroy bacteria, neutralize virus, and lyse red blood cells. It appears to act only in conjunction with complement and magnesium ion.(9)(13)(17)(24)(36)(48)(67)(68)(76)(113)

Chemical Nature of Antibodies

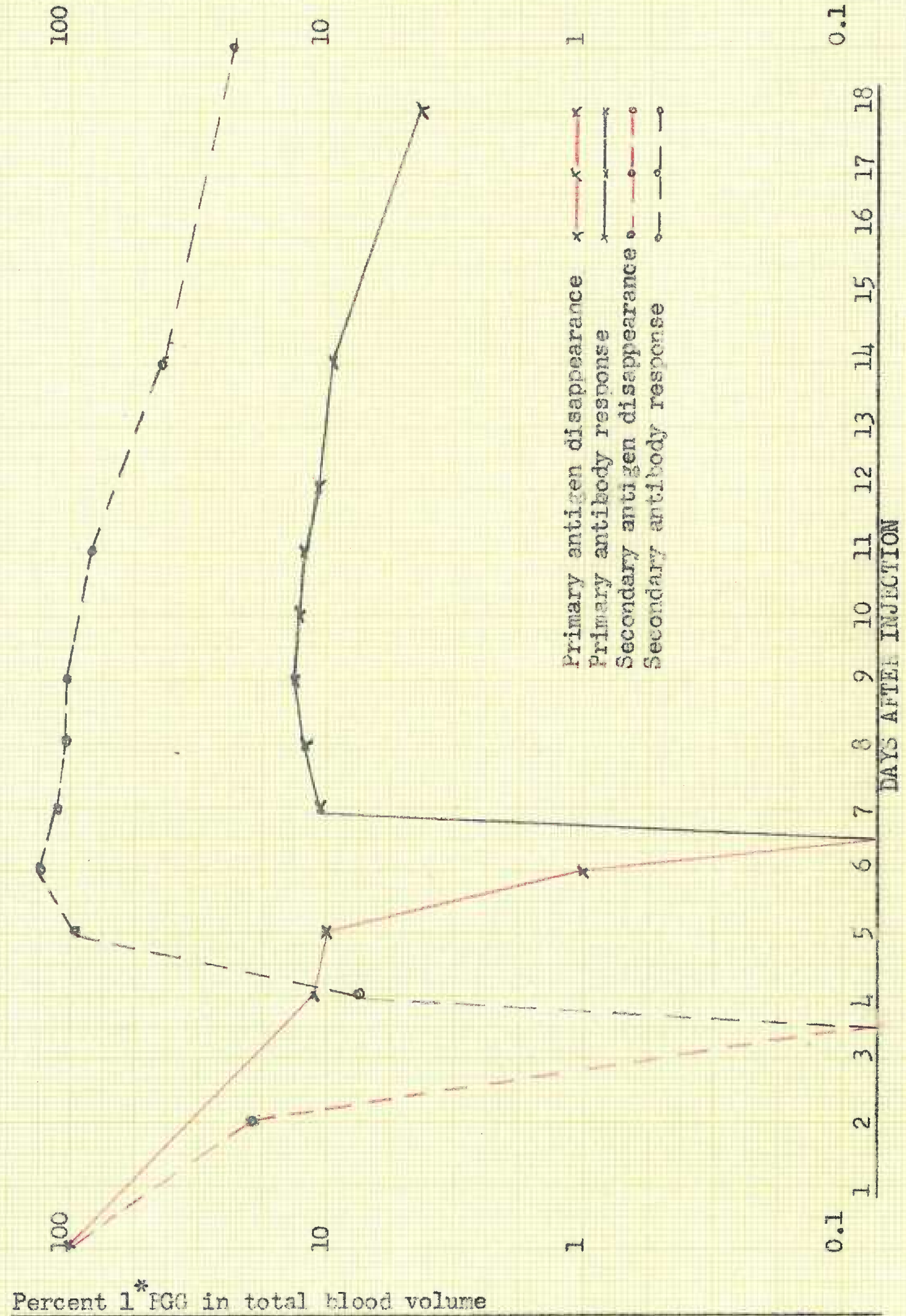
Antibodies are considered to be protein in nature or inseparably associated with protein for the following reasons: a) The activity of antiserum can be destroyed by heat at the same rate as proteins of the serum are denatured. b) Antibodies can be removed from antiserum by precipitation of the proteins. c) They can often be recovered in active form from such protein precipitates. d) They disappear from an immune serum acted upon by proteolytic enzymes in proportion to the extent to which hydrolysis is allowed to proceed. e) They appear to migrate with the gamma globulin fraction of the serum, or as a distinguishable component between the beta and gamma globulins.(17)(98) f) Chemical analysis reveals no uniform difference between antibody globulin and normal globulin. g) Immune gamma globulin can be fractionated, using partition chromatography to give a partial separation of an inert globulin from antibody.(101)

Animal Response to Antigen Injection

The primary and secondary reaction of an animal to the injection of antigen is shown on Table 1. Primarily injected antigen would be expected to disappear from circulating blood at a constant rate.

TABLE 1

JE 1*EGG precipitated by 1 ml serum at 60% antigen precipitation



On the fifth day however, it disappears at an accelerated rate and by the seventh day, is completely gone. This point on the curve corresponds with the appearance of circulating antibody.(5)(31)(49)(109)(110)(115)

The secondary response also shown on Table 1 illustrates the feature of the "recall" phenomenon, i.e. the antigen disappears sooner from the circulation; antibody is detected by the fourth day and reaches a higher titer because of increased plasma cell proliferation.(31)(82)(117) There is some evidence that antibody produced after secondary injection differs from that of primary injection in hemolytic activity and also in the partition chromatograph pattern.(46)(101)

The antibody which appears in the circulation represents only a small part of the total amount. Much is present in cells, tissue spaces and other body fluids. For example if antigen is injected into the footpad of a rabbit the lymph node draining this site will increase in size. The lymph node cells can then be transplanted and the recipient animal will show circulating antibody.(52)(53)(54)(55)(56)(57)(58)(59)(106)(135)

It is of interest that antibody is not released into the serum immediately following synthesis. At least one third of the total antibody protein needs to be synthesized before it can be detected in the circulating blood.(51) In connection with this release phenomenon the output of lymph appears to

increase preceeding the presence of antibody in the efferent vessel.(37)

Following parenteral injection, the spleen forms most of the antibody during the initial rise and stops at or about the peak titer. Splenic antibody then disappears and non-splenic sources take over the response at a slower rate. (128)(129)

There appears to be a correlation between antibody and plasma cell proliferation.(41)(42) Thus injecting a wax fraction of Mycobacterium tuberculosis causes a marked proliferation of plasma cells in lymphatic glands, spleen and liver, which in turn causes an increase in circulating antibody.(138) Persons with agammaglobulinemia are unable to make either plasma cells or circulating antibody.(128)

It is presumed that the antigen is phagocytized or absorbed by antibody forming cells located in various organs, i.e. lymph nodes, spleen, liver and lung.(128) Antigen has been labeled in such a way so as to be located within the cell into which it is absorbed.(110)(111). A fluorescent labeled antibody has been used to locate the antigen absorbing cell. From these studies antigen appears to be absorbed by large immature cells in the medullary area of lymph nodes draining the site of injection.(23)(30)(82)

Another technique for identifying the exact cell which produces antibody is with bacteriophages. An animal is immunized to a "T" phage of Escherichia coli. After antibody is produced,

single cells from the lymph nodes are removed and plated with the phage. Cells which appear to produce antibody can be subsequently isolated and removed with micropipettes. They appear to be of the plasma cell series.(3)

Amino Acid Synthesis

Spleen slices from immunized animals will incorporate radioactive amino acids into antibody protein during synthesis.(40)(69)(70)(72) With tissue cultures and C 14 labeled glycine it is found that the activity of the formed antibody could not be accounted for by non specific adsorption.(104)(105) Using this technique it has been shown that granulomatous tissue does not incorporate glycine into antibody as rapidly as the regional lymph node.(1)

Antibody appears to be synthesized de novo from the amino acid pool.(51)(130) Pantothenic acid deficient rats are unable to make antibody.(120) Low protein diets are also inhibitory.(12)(145)

The injection of oxidative metabolite inhibitors will also prevent antibody formation.(90)(117) Cortisone acetate has the ability to delay local antibody formation in the loose connective tissue of mice.(62)

Antibody Production by Various Organs

As indicated earlier, the lymph nodes and spleen play an important role in antibody production. The ability to synthesize antibody is not limited to these organs however. Many other tissues

are capable of carrying out this function although their efficiency appears to depend upon the number of plasma cells present.

In Table 2 the ability of various organs and cells to produce antibody has been summarized and designated as good or poor. The data have been arranged according to antigen used and the source is indicated by the reference number. Single and multiple injections were not separated. Unless stated otherwise rabbits were used; C stands for chicken; G stands for guinea pig; M stands for mouse; and R stands for rat.

The appendix, bone marrow, liver, lung, lymph nodes and spleen appear to be able to synthesize antibody under most conditions. The kidney was found to be a poor antibody producer in all cases except one; in this instance the adipose tissue of the renal sinus was used.(7)

Although the spleen is one of the principal antibody producers there were several instances where a poor response was obtained. Perhaps a difference in productivity between the red and white pulp may be responsible since the former contains more plasma cells.(23)

The liver has been found to synthesize antibody by some (6)(28)(35)(37)(85)(133)(138) and not by others (2)(4)(32)(47)(70)(94)(104)(119). It is known to make all of the alpha and beta globulins, but the gamma globulins are produced elsewhere.(88) Patients with chronic hepatic diseases are able to make three times as much

TABLE 2

ANTIBODY PRODUCTION BY VARIOUS ORGANS

GOOD	POOR
Red Blood Cells	
Bone marrow (35)(G 18)	Appendix (35)(127)
Liver (35)(127)	Kidney fat (35)
Lymph gland (G 18)	Lymph node (R 107)
Lymphocytes (37)(54)	Lymph plasma (54)
Spleen (114)(127)(129)(R 107)	Spleen (R 108)
	Subcutaneous tissue of lumbar region (35)
	Thymus (R 107)
Pneumococcus	
Adipose tissue of renal sinus (7)	Anterior wall of the stomach (28)
Bone marrow (28)(85)	Kidney (28)(103)
Draining lymph nodes (28)	Non injected skin (28)
Liver (6)(28)(85)(103)	
Lung (63)(85)	
Skin at site of injection (28)	
Spleen (6)(28)(85)(103)	
Globulin or Albumin	
Bone Marrow (73)(85)(119)(G 2)	Granulomatous tissue at site of injection (G 2)(G 137)
Granulation tissue around nodule at site of injection (137)	Kidney (119)(G 2)
Liver (6)(85)(G 138)	Liver (70)(119)(G 2)
Lung (85)	Lung (70)(119)(G 2)
Lymph Node (23)(33)(34)(70)(82)(106)(119)(136)(137)(G 2)(G 137)(G 138)	Lymph node (G 2)
Peritoneal cells (33)(34)	Macrophages (G 138)
Plasma cells in spleen red pulp (23)	Placenta (G 2)
Portal connective tissue of liver (23)	Thymus (32)(33)(G 2)
Spleen (70)(85)(86)(119)(121)(124)(G 2)(G 138)(G 147)	
Submucosa of ileum (23)	

TABLE 2 continued

Salmonella

Bone marrow (85)(132)(133)
Liver (6)(85)(133)
Lung (85)
Lymph nodes (38)(55)(104)(132)(M 87)
Lymphocytes (37)(54)
Lymph of Liver (47)
Peritoneal fluid (38)
Red pulp of spleen (42)(71)(132)(133)
Spleen (6)(85)(91)(134)
White pulp of spleen (132)

Bile (47)
Granulocytes of peritoneal exudate (38)
Kidney (104)
Liver (47)(104)(132)
Lymph follicles (42)
Lymph node (104)
Lymphocytes (71)(R 43)
Lymph plasma (54)
Macrophages (38)(105)(R 43)
Phagocytes (R 43)
Spleen (104)
Thymus (132)
Tissue at site of injection (38)
White pulp of spleen (71)

Toxin

Appendix (4)(116)
Bone marrow (119)
Cornea (96)
Fat (94)(95)(G 94)
Granulation tissue around nodule at site of injection (137)
Lymph nodes (82)(95)(116)(117)(119)(137)(G 137)
Mammary gland (4)
Muscle (94)(95)(G 94)
Skin (94)(G 94)
Spleen (4)(116)(117)(119)(R 120)
Uterus (4)
Vagina (4)

Bone marrow (94)(G 94)
Kidney (94)(119)(G 94)
Granulation tissue around nodule at site of injection (G 137)
Liver (4)(94)(119)(G 94)
Lung (119)
Spleen (94)(G 94)

Miscellaneous

Hog Cholera - Liver (65)
Vaccinia virus - Macrophages (60)
Brucella suis - Spleen (123)

antibody as normal persons. Synthesis appears to be a highly preferential function resulting in certain cases in a replacement of a large percentage of the globulin previously present. It appears to be operative at the expense of the production of serum albumin.(61) In patients with acute hepatitis there is no significant change in antibody production.(39)

The data in Table 2 suggest that either the cells capable of producing antibody are widely distributed or else they have the ability to migrate to various tissues as needed; the latter hypothesis is preferred. Finally, the animal, the antigen, the dose and the route of immunization all have some effect on the organ response.

Summary

1.) Antibody is composed of globulin molecules which are produced in regions of the body where reticulo-endothelial cells, lymphocytes, plasma cells and other relatively undifferentiated mesenchymal cells are aggregated.

2.) Antibody production following an antigenic stimulus rises to a peak and then diminishes, but continues at diminishing rate often for long periods.

3.) Antibody in the circulation is being constantly removed at a rate which is approximately proportional to its concentration.

4.) Antibody production continues after the antigen responsible has been eliminated from the circulation.

5.) A second or subsequent contact with the same antigen provokes a more rapid and active production of antibody (recall phenomenon).

6.) Antibody production is a function not only of the cells originally stimulated but of their descendants as well.

7.) The type of antibody production varies (a) according to the species used (b) with the age of the animal and (c) according to the nature and frequency of the antigenic stimulus. (12)

Antibody Production Theories

A general summary of theories on antibody formation would look something like this:

Theory	Proponents
I - SELECTION THEORIES	
(a) Complete pre-existance of the information Selection at a cellular level through common antigens or spontaneous mutation	(Ehrlich) (Burnet) (Talmage) (Lederberg)
(b) Selection at a molecular level involving successive steps of increased fitness	(Jerne)
II - INDUCTION THEORIES	
(a) Information is kept in the antigen during antibody production; "Template Theory"	(Breinl and Haurowitz) (Mudd) (Pauling) (Campbell)
(b) Information is transferred to autocatalytic intermediary units	(Burnet) (Schweet and Owen)
(c) Antigenic stimulation of genetic mutation	(Lederberg)
(d) Mixed Hypothesis -	
First step - induction of a specific structure	(Monod)
Second step - selection of the antigen	(Pappenheimer)

Table modified from (14)

The selection theories are those which have as their basic concept the presence in people of globulin which is able to act as antibody. This antibody (globulin) has relative specificity and some part of it will be able to react with any antigen. The antigen does the selecting at the cellular level, or finds the best antibody globulin and alters it so it can become a better antibody.(11)(12)(14)(64)(131) This second theory explains the differences between antibody which occurs naturally and antibody which appears after immunization.(64)

The induction theories consider that the antigen 1) either serves as a template from which antibody can be patterned or 2) transfers its imprint to DNA or RNA which in turn serves as the pattern. In either case antigen need not be present in the cell for antibody production to proceed.(11)(12)(14)(15)(92)(99)

In Lederberg's theory of antibody production he sets forth nine propositions, namely:

1.) Each antibody differs in the sequence of amino acids in the globulin portion. This is a relatively new notion as all previous theories agreed that antibody was unique in the folding of the protein but not in the order of amino acids.

2.) He has proposed a gene with the correspondingly characteristic sequence of nucleotides from which to pattern the antibody.

3.) Antibody forming cells are specialized. Their diversity arises from random mutations and these genotypes are maintained

throughout the life of the animal.

4.) During certain stages of cellular proliferation random assembly of the chromosomal DNA occurs. It would appear that faulty replicas of the globulin gene are initially fabricated and are then capable of exact, autonomous replication.

5.) Each cell, as it begins to mature, spontaneously produces small amounts of the antibody corresponding to its own genotype.

6.) To explain immune tolerance it is proposed that the immature cell is unusually susceptible to antigen-antibody complexes and that its proliferation is hereby controlled.

7.) The mature antibody forming cell is less reactive to antigen but will stimulate protein synthesis and the cytological maturation which mark a "plasma cell".

8.) Mature cells proliferate extensively under antigenic stimulation but are genetically stable and therefore generate large clones genotypically adapted to produce the homologous antibody.

9.) These antibody producing cells tend to persist after the disappearance of the antigen, retaining their capacity to react promptly to its later reintroduction.(81)

In the mixed hypothesis two steps of antibody formation are proposed. The first step is induction and implies the formation of a new specific structure on the cell surface adapted to the antigenic determinant. As a result of this process the induced cell could concentrate the inducing substance (immunogen) and by means of it, increase its antibody synthesis. The alternate theory

states rather that the induced cell will react to the "immunogen" by increased proliferation. In this way a clone is adapted to the synthesis of a certain type of antibody.(14)(98)

In conclusion it might be stated that the two main ideas vary only in how the cell acquires the mechanism for making antibody. The selection theories believing that antibody forming cells are present early in life and that specific stimulation by random antigens results in the increased production of a combination of naturally occurring globulins. The induction theories suggest that the antibody is made by a template, in the form of antigen, DNA, RNA or a gene; this template being formed by induction from the antigen or by spontaneous mutation.

COMPLETE AND INCOMPLETE ANTIBODIES

History

The discovery in 1900 by Landsteiner of human blood groups offered a solution to the reactions which sometimes occurred when whole blood was given a patient.(77) He thereby opened the way for the safe transfusion of blood. Despite the compatibility of the major and minor saline crossmatches, reactions still occurred. Particularly after patients had received several units of blood over a long period of time or after a woman had given birth to a jaundiced, or still-born (erythroblastotic) infant.(79)(83)(93)

In 1940 Landsteiner and Wiener established the presence of a new antigen in red blood cells. They found that if blood from a Rhesus monkey is injected into rabbits, the resulting antiserum will agglutinate the human cells of 85% of the population. This antigen, in common with the Rhesus monkey, was abbreviated to "Rh".(80)

Wiener and Peters subsequently demonstrated an antibody with Rh specificity in the serum of patients who had received multiple transfusions.(142) At almost the same time Levine and Stetson had described an antibody in the serum of mothers delivering erythroblastotic babies. This antibody was subsequently found to have Rh specificity.(83)

The theory of isoimmunization in pregnancy, as proposed by Levine and Stetson, demanded that antibodies be consistently

found against a component of the blood of the father. This component must also be present in the baby and absent from the blood of the mother. Actually in the early studies, such an antibody was infrequently present and often in low titers. This dilemma was solved by studies of Race and Wiener in 1944, who simultaneously demonstrated "blocking" antibodies. The serum in question was mixed with red cells containing Rh antigen and incubated. After a period of time, the excess serum was removed and a known anti-Rh agglutinating serum was added. If the cells did not clump it was assumed that they had been coated with an antibody already present in the unknown serum.(102)(139) The "blocking" antibody appeared to have the ability to coat cells and cover up antibody receptor sites, thus preventing the agglutinating antibody from attaching.(140)

In 1945, Diamond and Denton showed that "blocking" antibody could be made to agglutinate red blood cells if all of the reagents were prepared in 30% bovine albumin or in plasma instead of saline. (29) Shortly thereafter it was found that trypsinized red cells were agglutinated directly in saline by "blocking" antibody. The trypsin was able to alter the cell surface in some way but did not effect the haemagglutinogen loci.(89) More recently Pirofsky found that bromelin would also alter the cell surface and cause agglutination of red blood cells by "blocking" antibodies in saline.(100)

Another important method of showing blocking types of antibody was introduced by Coombs, Mourant and Race in 1945. Human "blocking" antibodies, as previously indicated, appear to be globulin in nature. Coombs produced a second antibody against this "blocking" globulin by injecting it into a rabbit. Thus when rabbit anti-human globulin was added to human red cells coated with "blocking" globulin, the cells agglutinated. This reaction is now known as the Coombs' test.(21)

Terminology

Because it was quite evident that the "blocking" antibody was not a "complete" one, i.e. incapable of agglutinating cells by itself, it was therefore termed "incomplete". It was also assumed that the "incomplete" antibody contained only one site which could combine with antigen and was therefore named "univalent". This was as opposed to the "bivalent" or "multivalent" antibodies, which posessed two combining sites and which would therefore, cause direct agglutination. Since the "incomplete" antibody will react best in albumin, it has also been termed "albumin" antibody.

Antibodies to the "A" and "B" blood group substances occur normally in serum of adults and are sometimes referred to as "natural" antibodies. Those to the Rh antigen occur only after immunization and are "immune" antibodies.

For the rest of this thesis the term "incomplete" will refer to the "blocking","univalent","albumin" or "immune" antibodies, since they appear to be one and the same antibody.(67)

Properties of "Incomplete" Antibodies

The discovery of antibodies of "incomplete" variety stimulated considerable interest concerning their characterization. Wiener believed that "incomplete" antibodies were smaller in molecular size than "complete" antibodies since they passed the placenta. (141)(143)(144)(148)

Using a dialysis technique Witebsky and others were able to separate "complete" and "incomplete" antibodies in 1946. The "incomplete" antibody stayed in the supernatant fluid which contained the albumin fraction and a certain amount of the globulin. The "complete" antibody was in the precipitate which was the globulin fraction.(146)

"Complete" antibodies usually are affected by acid, while "incomplete" antibodies are not.(67) They will also react better at low temperatures, i.e. approximately 4°C. The "incomplete" antibodies react equally well or better at 37°C. When titrations for "incomplete" antibodies are incubated at 37°C, 20°C and 5°C the proportions of positives at each temperature are 99:63:71.(25)

"Complete" antibodies are also destroyed by heating for 5-10 minutes at temperatures between 65 and 70°C. The "incomplete" antibodies appear not to be so affected. The "incomplete"

antibodies are absorbed perferentially to red blood cells and can be eluted easily as opposed to the "complete" antibodies. The above work was first done by Coombs and Race in an effort to characterize "incomplete" antibody and subsequently confirmed by Sturgeon.(22)(126)

Other Forms of "Incomplete" Human Antibodies

As more and more was being learned about the varieties of Rh antigens and antibodies other related fields were also subject to study. Loveless and Cann were able to demonstrate an unusual antibody in cases of allergy to ragweed pollen. As a result of their work two types of antibodies are now associated with allergy; they have been separated and characterized by electrophoresis. The beta globulin fraction is concerned with sensitization and is termed "reagin". Following immunization a gamma globulin fraction appears, which is capable of blocking the activity of reagin. Needless to say there is some overlapping of the two fractions. It was of interest that this type of "blocking" antibody had a lower mobility then the reagin.(16)(84) Various workers have attempted to demonstrate the blocking type of antibody of Loveless and Cann *in vitro*. Boyden suggested adsorbing antigen on tannic acid treated red blood cells. The coated cells would then be agglutinated by "incomplete" or "non-precipitating" antibody.(10) Variable results have been obtained by others and in most instances the test was not successful.(8)(19)(97)(112)

Coombs suggested a modification of the Boyden test, which includes the use of an antiglobulin serum of the type discussed with the Rh fraction.(20)

Kuhns injected diphtheria toxoid into humans and found that two types of antitoxin appeared, 1.) a form which precipitated with homologous toxin; 2.) a type which failed to precipitate but which was capable of sensitizing skin. These varieties of antitoxin could be separated by starch electrophoresis. The precipitating fraction appeared to move slowly in the gamma² fraction and the non-precipitating portion moved faster in this fraction.(74) Further studies subdivided the non-precipitating globulin into skin-sensitizing and non-skin-sensitizing components.(75) It was of interest that an allergic family history was associated with the ability of the subject to produce the skin sensitizing globulin.(36)

It has been noted by several workers that antibodies can be produced against insulin. They will react with the insulin in such a way that it no longer is able to control the diabetes. The antibody is of the "blocking" or "incomplete" type as it will neutralize the enzymic action of insulin but will not precipitate it from solution.(16)(84)(98)

Systems of "Incomplete" Antibodies in other Animals

Not all the work on "complete" and "incomplete" antibodies has been done in humans. As indicated earlier quite a bit of work has been done in animals to determine the site of antibody

production. In most instancesthe nature of the antibody was not studied.(Table 2)

Gorer was able to induce a hemagglutinin response in mice as a result of inoculation of heterologous mouse tumors. He demonstrated that two types of antibodies were produced. One was reactive in saline and the other was evident in an environment consisting of 50% serum. Gorer's work was largely concerned with tissue transplantation in genetically defined strains of mice. He did call attention, though, to the analogy between his findings and the Rh-Hr system.(50)

Other workers, Kaliss, Hoecker, Bryant, Foley, Morgan and Greco used this antigen-antibody system, i.e. the ability of mice to be isoimmunized, in tests on the effect of cortisone and other drugs.(45)(66) Undoubtedly others have also used this system.

Frisch and Davies have reported work with mice using sheep, rabbit and human red blood cells as antigen. They were able to demonstrate a marked difference in saline andalbumin titers. They injected mice with 0.5 ml of packed human group O DCe cells on days, 1,2,3,15 and 16. On day 44, they injected 0.5 ml of cells subcutaneously and bled the animals from the heart 7 days later. The titer of saline antibody was 10^{-4} and of albumin, 10^{-7}. After refrigeration the titers were of approximately the same height. Further work on varying dose was done. The results again indicated a higher albumin then saline titer. With smaller

doses, i.e. 0.005 or 0.0005 ml the saline antibody was completely negative while the albumin antibody reached a minimum titer of 1:512 after 8 days. With a dose of 0.05 ml, differences of 1:1024 (saline) to 1:131,172 (albumin) were obtained.(48)

The conclusions drawn from this experiment were that injection of human, sheep and rabbit red blood cells into Webster mice resulted in the appearance of an "incomplete" antibody. This "incomplete" antibody could be present in quite high titers and would not be detected by conventional methods. As a result of this observation a tool was now available for the detailed study of antibodies of the "incomplete" type.

The purpose of this thesis is to present additional data on those factors which might favor the production of "complete" or "incomplete" antibodies in mice as follows: 1. route of injection of antigen; 2. dose of antigen; 3. age of antigenic cell; 4. number of injections of antigen; 5. diet; 6. temperature; 7. strain differences.

It was also hoped that the sites of "complete" and "incomplete" antibody production could be established. The system to be used was that of removal of organs from immunized donors, mincing them and injecting them into non-immune recipients. By bleeding the recipient animals the comparative rate of production and release of "complete" and "incomplete" antibodies could be determined. Among the organs to be studied were spleen, liver and lung.

MATERIAL AND METHODS

Antigens

The antigens used were human and sheep red blood cells. The human cells were obtained from units of whole blood in ACD solution, given us by the Red Cross and Clinical Pathology, or blood drawn from members of the department, using Alsiever's solution as anticoagulent. All human blood was of the type O Rh "D" positive. The sheep red cells were, from a pool of several animals, obtained from Clinical Pathology.

Animals

Webster female albino mice of varying ages were used. Unless indicated, they were at least five weeks old, fed on Purina mouse food and kept at room temperature. Usually groups of three to six animals were used.

Injections

Blood for injection was washed three times with physiological saline. It was then packed for three minutes at approximately 3000rpm and diluted 1:2. A dose of 0.1 ml, i.e. the equivalent of 0.05 ml packed cells, was given intraperitoneally unless otherwise indicated.

Bleedings

Mouse serum was obtained by bleeding the mice from their tails or from the orbital sinus.(125) The blood was collected in silicon coated tubes by the former method or capillary tubes by the latter. The blood was allowed to clot, tubes spun and serum removed within

twenty-four hours.

Dilutions

Dilutions were made in physiological saline or 5% human albumin in physiological saline. Serum was first diluted with physiological saline using capillary pipettes and counting the drops of serum used in proportion to the drops of saline. Then 0.2 ml of this mixture was transferred to a tube containing 0.2 ml of saline or 5% albumin and further two-fold dilutions made.

Trypsinization

In order to trypsinize the red cells a 5% suspension of washed cells was incubated at 37°C with 1% trypsin for 10 minutes. After incubation the cells were washed with physiological saline another three times before a 2% suspension of packed cells was prepared and used for the titrations.

Titrations

One drop of a 2% suspension of either washed or trypsinized and washed red blood cells, was added to each tube and allowed to incubate for at least one hour at room temperature. The tubes were then spun at approximately 3000 rpm in a small international centrifuge for approximately two minutes. The last dilution still containing readily visible clumps of cells, after shaking, was taken as the end point and its titer used as the titer of the serum. In all instances, unless indicated, a negative result represents no agglutination at a titer of 1:8.

Coombs' Test

An anti-mouse globulin sera was obtained by injecting normal mouse serum into a rabbit and absorbing the rabbit's serum with human red blood cells. Before adding the anti-mouse globulin sera to the three saline titration tubes beyond the end point, the cells were washed with 0.5 ml physiological saline. After addition of the saline the tubes were spun at approximately 3000 rpm for 2 minutes, the saline removed and one drop of 1:100 dilution of anti-mouse globulin serum added. The tubes were then allowed to stand for a minimum of twenty minutes before they were again spun for two minutes, read and graded as indicated previously.

RESULTS

Titer Variations - Individual Mice

In Table 3 are shown the results of a test in which mice were immunized with the standard antigenic dose intraperitoneally. They were tested after 9, 16 and 24 days. Titers from individual mice are shown together with the average and also the average difference. For any particular bleeding and variety of titration the use of three of six animals in a group could be expected to yield similar results. Two-fold differences in titrations are not considered to be significant, but a four-fold difference might well be significant. It may also be observed that "incomplete" antibody, as evidenced by elevated albumin and Coombs titers, were not outstanding in animals stimulated with a single dose of antigen.

Method of Bleeding

Two variables were studied in Table 4 using three mice in each group. The variables include bleeding from the cut tail versus from the orbital sinus and silicon coated versus non coated tubes. In this case animals received multiple standard injections intraperitoneally, in an effort to obtain high titer sera. Examination of the Table reveals titers almost identical in each group of mice. It would appear that the source of blood and the manner of its collection did not affect the result.

Influence of Contact with Clot

During the course of some experiments it was thought the

TABLE 3

Titration Variations - Individual Mice

HUMAN CELLS

Mouse	Bleeding No. 1			Bleeding No. 2			Bleeding No. 3		
	Sal.	Alb.	C.T.	Sal.	Alb.	C.T.	Sal.	Alb.	C.T.
1	128	256	256	256	512	256	512	512	512
2	64	128	128	128	128	128	32	64	128
3	32	64	64	32	32	32	64	128	64
4	64	512	256	32	64	32	32	32	32
5	128	512	256	128	256	128	128	256	128
6	128	128	128	256	512	256	32	128	32
7	32	128	32	64	128	64	64	32	32
8	32	256	256	64	256	64	128	256	128
9	64	64	128	256	512	256	256	256	256
Aver.	75	227	167	135	266	135	145	185	144
Av. Dif.	35	139	79	80	163	80	112	120	105

SHEEP CELLS

Mouse	Bleeding No. 1			Bleeding No. 2		
	Sal.	Alb.	C.T.	Sal.	Alb.	C.T.
1	128	64	64	32	16	32
2	32	32	32	128	32	128
3	256	128	256	256	256	256
4	256	256	256	64	128	64
5	256	256	256	128	128	128
6	128	64	128		expired	
Aver.	176	133	165	121	110	121
Av. Dif.	80	81	90	59	70	59

The numbers 16, 32, 64, 128, 256 and 512 represent the reciprocal of the greatest dilution of the original serum which showed a distinct agglutination reaction, i.e. 1:16; 1:32; 1:64; 1:128; 1:256 and 1:512.
Sal. - Saline titer; Alb. - Albumin Titer; C.T. - Coombs' titer.
Aver. - Average titer; Av. Dif. - The average difference in titers.

TABLE 4

Method of Bleeding

Group A Animals

High titer mice bled from tail into silicon coated tube:

Saline - 32768
Albumin - 131072
Coombs - 65536

High titer mice bled from orbital sinus into clean capillary tube:

Saline - 32768
Albumin - 131072
Coombs - 65536

Group B Animals

High titer mice bled from orbital sinus into silicon coated tube:

Saline - 32768
Albumin - 524288
Coombs - 262144

High titer mice bled from tail into clean capillary tube:

Saline - 32768
Albumin - 524288
Coombs - 262144

time of removal of mouse sera from the clot might affect the titer. The results of an experiment to test this notion are presented in Table 5. In this experiment a pooled sample of bleod from ten mice, who had previously received multiple injections of red blood cells intraperitoneally, was used. Time of contact with the clot appears to have had no influence on the subsequent albumin and Coombs' titers.

Temperature and Time

Among the factors which were tested were effects of temperature and the time of incubation. The data from one experiment, in which dilutions of a single pooled serum were used, are presented in Table 6. It can be noted that titers remain unchanged irrespective of the length of time of incubation. Secondly, albumin titers appear to be insensitive to temperature variation at 5°C, only a two-fold reduction being observed. On the other hand the saline titer was definitely decreased eight-fold by lowering the incubation temperature to 5°C.

In the usual experiment one hour incubation was used and tubes were kept at room temperature, i.e. 26-30°C.

Stroma and Hemoglobin

From the results as shown in Table 7 it can be seen that the antibody response is to the red blood cell stroma and not the hemoglobin. The addition of hemoglobin to stroma augmented the titer. This observation was not studied further.

TABLE 5

Influence of Contact with Clot

Serum removed immediately after blood clotted:

	TIME SERUM AT 4°C BEFORE TITRATION				
	0 Hrs.	24 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.
Saline	1024	2048	1024	1024	1024
Albumin	16384	16384	16384	65536	32768
Coombs	4096	8192	2048	2048	2048

Serum remaining on clot for time indicated and removed just prior to titration:

	TIME SERUM ON CLOT AT 4°C BEFORE TITRATION			
	24 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.
Saline	2048	2048	2048	2048
Albumin	8192	8192	32768	16384
Coombs	4096	2048	4096	4096

TABLE 6

Temperature and Time

SALINE

Incubation Temperature	READINGS MADE AFTER INCUBATION FOR: 1 Hr.	$1\frac{1}{2}$ Hrs.	2 Hrs.	$2\frac{1}{2}$ Hrs.	3 Hrs.	$3\frac{1}{2}$ Hrs.
5°C	1024	1024	1024	1024	1024	1024
26°C	8192	8192	8192	8192	8192	8192
37°C	4096	8192	8192	8192	8192	8192

ALBUMIN

Incubation Temperature	READINGS MADE AFTER INCUBATION FOR: 1 Hr.	$1\frac{1}{2}$ Hrs.	2 Hrs.	$2\frac{1}{2}$ Hrs.	3 Hrs.	$3\frac{1}{2}$ Hrs.
5°C	8192	8192	8192	8192	8192	8192
26°C	16384	16384	16384	16384	16384	16384
37°C	16384	16384	16384	16384	16384	16384

TABLE 7

Stroma versus Hemoglobin

ANTIBODY	DAYS AFTER INJECTION					
Saline	7	19	25	32	46	62
RBC	64	8	32	32	0	0
Stroma	16	8	8	64	32	0
Hemoglobin	0	0	0	0	0	0
St. and Hb.	64	16	64	128	128	32
Albumin						
RBC	128	32	32	64	16	0
Stroma	64	32	16	256	32	0
Hemoglobin	0	0	0	0	0	0
St. and Hb.	128	128	128	256	256	32
Coombs						
RBC	64	32	32	64	0	0
Stroma	16	64	32	128	32	0
Hemoglobin	0	0	0	0	0	0
St. and Hb.	128	64	64	256	128	32

RBC - Red Blood Cells; St. - Stroma; Hb. - Hemoglobin

Age of Cells

Red cells do not appear to lose their antigenicity on standing, nor do they produce higher albumin and Coombs' titers. As observed in Table 8, the antigenicity of red cells was tested both by injecting old and new cells and by titering the same serum with old and new cells.

Dose and Route

In a previous experiment, Frisch and Davies had demonstrated a significant albumin response to as little as 0.005 ml of packed blood cells given subcutaneously. This experiment was repeated using intraperitoneal and subcutaneous routes with doses varying from 0.05 to 0.0005 ml of cells. In this experiment only the 0.05 and 0.005 ml intraperitoneal doses and the 0.05 ml subcutaneous dose stimulated antibody rise but no significant difference between"complete" and "incomplete" response was noted. These discrepancies have not, as yet, been explained.

Consecutive Doses

In an effort to separate the "complete" from the "incomplete" antibody another group of animals was given single versus two, three and four consecutive standard doses of antigen intraperitoneally. Similarly this increased dosage was not reflected in a significant rise of albumin and Coombs' titers.

Diet

The problem of diet was approached in a crude way. For this purpose, groups of mice were placed on diets as shown on Table 9

TABLE 8

Age of Cell

3 month old cells injected and titered

	DAYS AFTER INJECTION		
	7	14	21
Sal.	8	32	32
Alb.	64	64	64
C.T.	8	ND	ND

3 month old cells injected and new cells titered

	DAYS AFTER INJECTION		
	7	14	21
Sal.	32	32	32
Alb.	64	128	64
C.T.	32	32	32

1 day old cells injected and titered

	DAYS AFTER INJECTION		
	7	14	21
Sal.	8	8	64
Alb.	16	32	128
C.T.	8	32	64

1 day old cells injected and old cells titered

	DAYS AFTER INJECTION		
	7	14	21
Sal.	4	16	16
Alb.	8	32	32
C.T.	4	ND	ND

Sal. - Saline Titer; Alb. - Albumin Titer; C.T. - Coombs' Titer; ND - Not done.

TABLE 9

Diet

SALINE

Diet	DAYS AFTER PRIMARY STIMULUS 9	14	21	28	SECONDARY STIMULUS ON DAY 29	DAYS AFTER SECONDARY STIMULUS 6	13	20
Bread	16	8	4	16		256	128	256
Meat	128	32	32	16		256	256	ND
Dog Bis.	512	128	128	64		4096	256	512
Mse Bis	128	128	128	256		2048	512	512

ALBUMIN

Diet	9	14	21	28		6	13	20
Bread	32	32	8	32		256	128	512
Meat	128	64	32	64		1024	256	ND
Dog Bis	512	256	256	128		4096	256	1024
Mse Bis	128	128	128	256		4096	2048	1024

COOMBS

Diet	9	14	21	28		6	13	20
Bread	16	32	16	16		512	ND	512
Meat	256	128	32	16		2048	ND	ND
Dog Bis	1024	256	128	64		8192	ND	1024
Mse Bis	256	512	512	512		2048	ND	1024

ND - Not done; Dog Bis - Dog Biscuits; Mse Bis - Mouse Biscuits

for three days before they were immunized with the standard dose, bled at intervals and titers obtained. A second standard dose of antigen was administered intraperitoneally approximately one month later.

A study of the Table reveals a decrease in the response to primary antigen in the animals receiving the bread (low protein) diet. This was expected from the results obtained by Wissler and associates.(145) Even more important was the fact that extremes of diet did not appear to alter the ratio of "complete" and "incomplete" antibody as was hoped.

<u>Temperature</u>

In an effort to stimulate a spread between saline and albumin antibody, animals were placed at the following temperatures 5°C, 26°C and 37°C, immediately after immunization, and bled after 4, 11 and 18 days. Surprisingly, no inhibition at the cold temperature occurred nor was there an increase at the high te temperature. Again there was no change in the saline and albumin ratio. No attempt was made to measure the actual body temperature of the mice. One can only assume that the regulatory mechanism functioned properly. The animals were stressed as shown by the fact that over half died at the two extreme temperatures.

<u>Inactivation of Serum</u>

Sera obtained from animals, which had received multiple standard injections of antigen, were inactivated by heat at various temperatures and for various lengths of time. The

results obtained are set forth in Table 10. Heat does not appear to selectively destroy either the saline or albumin antibody. The reduction in titer, after heating at high temperatures, could be explained by the fact that the respective sera did coagulate and were not easily resuspended.

In instances where titrations were begun with dilutions of 1:2, hemolysis occurred up to 1:8. This hemolysis was absent if the serum was first heated in such a way so that complement was destroyed.

Strain

In previous unreported studies of Frisch and Davies, they had observed no significant difference in saline and albumin antibodies in various strains of mice. Characteristic strain patterns have been observed by others.(26)(27)(44)(122)

For reasons which will be discussed later three varieties of Webster mice were given the standard antigenic dose and titers obtained as indicated in Table 11. Although differences in response were observed there were no changes in the "complete" and "incomplete" antibody ratio among the three strains.

Natural antibody

In an attempt to explain "incomplete" types of antibody, which are likely to be produced under some conditions and not others, it was suggested that the presence or absence of natural antibody for the particular antigen might be the determinant

TABLE 10

Inactivation of Serum

SALINE

Temperature for 5 minutes	Titer
60-65°C	1024
65-70°C	256
70-75°C	16
75-80°C	0
Control	2048

ALBUMIN

Temperature for 5 minutes	Titer
60-65°C	8192
65-70°C	512
70-75°C	32
75-80°C	16
Control	16384

COOMBS

Temperature for 5 minutes	Titer
60-65°C	1024
65-70°C	256
70-75°C	16
75-80°C	0
Control	2048

TABLE 11

Strain Difference

SALINE

Strain	DAYS AFTER INJECTION 6	21	28	33	40	53	69
NIH	256	128	8	32	16	128	128
Inbred	128	128	16	128	64	64	ND
Webster	64	128	4	64	16	4	ND

ALBUMIN

Strain	6	21	28	33	40	53	69
NIH	512	128	8	32	32	128	128
Inbred	256	128	32	128	128	128	ND
Webster	64	128	8	128	32	4	ND

COOMBS

Strain	6	21	28	33	40	53	69
NIH	512	256	8	64	16	128	128
Inbred	128	128	16	128	64	128	ND
Webster	64	128	16	64	16	32	ND

NIH - National Institute of Health original Webster strain
Inbred - Webster inbred strain
Webster - Webster strain regular
ND - Not done

factor. In addition while naturally occurring hemagglutinins for various species have been described, no one has specifically searched for naturally occurring "incomplete" antibodies with the above points in mind.(26)(27)(44)(122) Testing a pooled serum derived from normal Webster mice against a variety of red blood cells gave the results shown on Table 12.

As expected, mouse sera contained normal antibodies for human, rabbit, rat and hamster. They were absent against cow, dog, guinea pig, goat, sheep, frog and iguana.

The antibodies, when present, were demonstrated in saline and albumin but not in titers significantly different. The only exception was observed with the albumin antibody to human red blood cells, which was present in titers of 1:20 or more, while the saline antibodies were almost always absent. In addition the presence or absence of antibody was not correlated with ABO or Rh type blood.

Transplantation

On August 14, 1958 Dr. Frisch and Mr. Davies performed an experiment in which the liver, lungs and spleen of seven week old mice, injected with 0.05 ml of packed human red cells on July 7, 1958, were removed and ground up separately in the Waring blendor with 10 ml of physiological saline for two minutes. The cell suspensions were centrifuged for three minutes at approximately 2000 rpm on a large International

TABLE 12

Natural Antibody

238 Samples of Human Blood tested		8 Samples of Rabbit Blood
SALINE -	1:10 6% positive	1:10 100% positive
	1:20 0.4% positive	1:20 100% positive
	1:40 100% negative	1:40 75% positive
ALBUMIN *	1:10 87% positive	1:10 100% positive
	1:20 76% positive	1:20 100% positive
	1:40 13% positive	1:40 100% positive
COOMBS -	1:10 16% positive	1:10 100% positive
	1:20 5% positive	1:20 100% positive
	1:40 2% positive	1:40 43% positive

10 Samples of Rat Blood tested		1 Sample of Hamster Blood
SALINE	1:10 100% positive	1:10 100% positive
	1:20 100% positive	1:20 100% positive
	1:40 100% positive	1:40 100% positive
	1:80 60% positive	
ALBUMIN	1:10 100% positive	1:10 100% positive
	1:20 100% positive	1:20 100% positive
	1:40 100% positive	1:40 100% positive
	1:80 100% positive	
	1:160 100% positive	
	1:320 2% positive	
COOMBS	1:10 100% positive	1:10 100% positive
	1:20 100% positive	1:20 100% positive
	1:40 100% positive	1:40 100% positive
	1:80 44% positive	
	1:160 10% positive	

25 Samples of Cow Blood tested - all dilutions negative
10 Samples of Dog Blood tested - all dilutions negative
10 Samples of Guinea Pig Blood tested - all dilutions negative
1 Sample of Goat Blood tested - all dilutions negative
1 Sample of Sheep Blood tested - all dilutions negative
1 Sample of Frog Blood tested - all dilutions negative
1 Sample of Iguana Blood tested - all dilutions negative

centrifuge and resuspended in physiological saline. This washing process was repeated two more times and the cells were then injected into groups of mice in such a way that the animals received the equivalent, by volume of 1 whole organ, 1/5, 1/10, or 1/50 of an organ.

The results of this experiment are shown on Table 13. The titration values obtained would lead one to believe that the cells which produce albumin (incomplete) antibodies could be separated from those which were saline antibody producers, i.e. 1/5 liver intraperitoneally and 1/50 lung subcutaneously. Accordingly, the above experiment was repeated.

A total of 0.05 ml packed sheep cells were injected into mice on May 8, 1959. These animals received a secondary stimulus of 0.1 ml packed sheep cells on June 10, 1959. On June 18, 1959 the animals were sacrificed by decapitation and their spleens removed. The spleens were placed in a Waring blendor for two minutes; material then collected and washed three times with physiological saline, centrifuging each time for ten minutes at approximately 3000 rpm. Dilutions equivalent to 1/10 and 1/100 spleen were made and injected. Titrations were done 4, 11, 18, 25 and 32 days after transplantation. In this experiment antibodies were not demonstrated in any of the groups of animals tested.

In the second experiment the factor of primary versus secondary stimulization was studied. It had been assumed that

TABLE 13

August 14, 1958 Transplantation

SALINE

Organ	Route	Days after transplantation 4	6	12	29	65
1 Spleen	I.P.	0	0	0	128	32
1/5 Spleen	I.P.	0	0	0	0	ND
1/10 Spleen	I.P.	0	32	16	512	512
1/50 Spleen	I.P.	64	0	0	0	0
1 Spleen	S.C.	0	0	0	0	0
1/5 Spleen	S.C.	0	0	0	0	0
1/10 Spleen	S.C.	0	0	0	0	0
1/50 Spleen	S.C.	0	0	0	0	0
1 Liver	I.P.	512	256	256	0	ND
1/5 Liver	I.P.	0	0	8	0	ND
1/10 Liver	I.P.	0	0	0	0	ND
1/50 Liver	I.P.	0	0	0	0	ND
1 Liver	S.C.	0	0	0	0	0
1/5 Liver	S.C.	0	0	0	0	0
1/10 Liver	S.C.	0	0	0	0	0
1/50 Liver	S.C.	0	0	0	0	0
1 Lung	I.P.	0	0	0	0	ND
1/5 Lung	I.P.	0	0	0	0	ND
1/10 Lung	I.P.	0	0	0	0	256
1/50 Lung	I.P.	0	0	0	0	0
1 Lung	S.C.	0	0	0	0	0
1/5 Lung	S.C.	0	0	0	0	512
1/10 Lung	S.C.	256	2048	128	128	1024
1/50 Lung	S.C.	0	0	16	0	0

TABLE 13 continued

ALBUMIN

1 Spleen	I.P.	16	16	32	4096	256
1/5 Spleen	I.P.	0	0	0	8	ND
1/10 Spleen	I.P.	2048	32768	16384	32768	2048
1/50 Spleen	I.P.	4096	8192	8192	16384	32
1 Spleen	S.C.	0	64	16	1024	0
1/5 Spleen	S.C.	16	0	0	16	128
1/10 Spleen	S.C.	0	0	0	512	0
1/50 Spleen	S.C.	0	0	0	32	32
1 Liver	I.P.	32768	65536	65536	0	ND
1/5 Liver	I.P.	4096	2048	1024	2048	ND
1/10 Liver	I.P.	16	0	8	16	ND
1/50 Liver	I.P.	0	0	0	128	ND
1 Liver	S.C.	8	0	0	64	0
1/5 Liver	S.C.	0	0	0	0	0
1/10 Liver	S.C.	0	0	0	0	0
1/50 Liver	S.C.	0	0	0	0	0
1 Lung	I.P.	0	32	16	0	ND
1/5 Lung	I.P.	0	0	0	0	ND
1/10 Lung	I.P.	1024	64	0	0	256
1/50 Lung	I.P.	0	0	0	0	64
1 Lung	S.C.	16	32	0	0	256
1/5 Lung	S.C.	0	128	8	0	512
1/10 Lung	S.C.	32768	4394304	32768	4096	1024
1/50 Lung	S.C.	2048	2048	1024	128	0

I.P. - Intraperitoneal injection
S.C. - Subcutaneous injection
ND - not done

the cells would colonize in the recipients and continue to produce antibody. No attention had been paid to the way in which the donor animals were injected. In the original experiment by Frisch and Davies the donor mice had received a single injection of antigen approximately one month previously. In the experiment reported above the donor animals had been given a second antigenic stimulus just prior to transplantation. The significance of more than one antigen injection was tested in an experiment as follows:

Animals who received a primary injection of sheep cells on May 20, 1959 were used as donors on July 23, 1959, approximately two months later. Another group of these same mice received a second antigenic stimulus of 0.1 ml packed sheep cells on July 22, 1959. Transplantations from this group of animals were done on July 24 and again on July 29. At the time of actual transplantation the animals were decapitated and blood specimens were collected. The spleens were removed and washed three times with physiological saline. In this experiment the recipients were given intact splenic tissue or standard homogenized tissue as follows: a) whole spleen, b) 1/4 spleen, c) 1/8 spleen. The recipient animals were bled 5, 12, 19 and 26 days after transplantation.

The results of the experiment did not duplicate those obtained by Frisch and Davies. As a matter of fact, antibody in the recipients appeared only in those animals given an intact spleen from secondarily stimulated donors. The data obtained from these recipients are presented in Table 14. Antibody titer

TABLE 14

July 24, 1959 and July 29, 1959 Transplantations

Mouse	Days after Transplantation				Stimulus on day 33	Days after Stimulus		
	5	12	19	26		6	13	22
SALINE								
1	32	0	0	0		128	expired	
2	16	0	0	0		512	128	128
3	256	64	0	0		256	256	64
4	128	32	8	0		512	1024	2048
Controls						512	1024	512
ALBUMIN								
1	64	0	0	0		256	expired	
2	0	0	0	0		256	128	128
3	256	16	0	0		128	256	128
4	256	32	16	0		1024	4096	2048
Controls						256	2048	512
COOMBS								
1	64	0	0	0		256	expired	
2	0	0	0	0		512	1024	128
3	256	64	0	0		256	1024	64
4	128	32	16	0		4096	1024	2048
Controls						4096	8192	512
TRYPSIN								
1	32	0	0	0		256	expired	
2	16	0	0	0		512	128	128
3	256	32	0	0		256	256	64
4	64	32	8	0		1024	2048	2048
Controls						1024	1024	512

decreased so rapidly after transplantation that it was probably derived from passive transfer. To test this hypothesis, the recipient animals were given a dose of 0.05 ml packed sheep cells approximately one month after transplantation. The antibody response after this immunization was of a primary type which suggests that the donor cells had not survived in the recipient animals and that the original antibody had been transferred passively.(Table 14)

In the third experiment the factor of excessive heating of the Waring blendor during its operation was investigated. In this experiment animals primarily immunized against sheep or human cells on July 31, 1959 were used as donors. On August 21, 1959 they were decapitated, spleens removed and ground in the Waring blendor without an ice jacket, as done originally, and with an ice jacket. In the former instance the temperature of the contents was 35°C, in the latter it was 16°C. The homogenates were treated as in the original experiment and given to recipient mice in varying doses. Mice were bled and titered after 3,7,15 and 19 days. The failure of the cells to survive and to produce antibody did not appear to be due to heating. In the above experiment antibody was not demonstrated in any of the blood samples obtained from recipient mice.

A review of the protocol suggested that an important variable might be the duration of time between primary stimulus and organ transplantation. It was felt that possibly a period of maturation was required in order to successfully transplant cells

to recipients. This hypothesis was tested with the fourth experiment by removing spleens, lungs and livers, 10, 20, 30, and 40 days after primary stimulation. Organs were homogenized in the Waring blendor, washed with saline and amounts equivalent to 1/4 and 1/20 of an organ were injected intraperitoneally and subcutaneously. The animals were bled 7, 14, 18 and 24 days after transplantation. Antibodies were not demonstrated in the serum of the recipient mice.

In the fifth experiment variation in age of the donor animal was investigated. For this purpose two to three, five to six and eight to nine week old mice were injected with human blood on November 16, 1959. On December 8, 1959 they were decapitated, the spleens removed and homogenized in the Waring blendor or minced with a knife. The equivalent of 1/4 of an organ was injected. The mice were bled 4, 11, 18 and 25 days after transplantation. The age of the donor animals did not appear to influence the transplantation since none of the recipient animals formed antibodies.

In the original experiment by Frisch and Davies the recipient mice were believed to have received high protein dog biscuits rather than the standard mouse wafer. In experiment six this factor was examined. Recipient animals received the equivalent of 1/4 of a minced spleen from donors with high antibody titers. One group of recipients was fed on high

protein dog biscuits and another group on Purina mouse food. Titrations were done after 4, 11, 18 and 24 days. Despite the high antibody titer in the donor (Saline 1:5120, Albumin 1:20480) the recipient mice failed to develop antibody.

For all the experiments done in the laboratory the mice had been obtained from a supplier in California. The purchase order usually specified "Webster" mice of a certain age and sex. An inquiry by mail concerning possible changes in the strain or unusual illness in the animals since July 7, 1958 revealed the following: the supplier maintained three separate strains of Webster mice; 1) NIH 2) Inbred 3) Webster. Unless specified to the contrary an order for "Webster mice" was filled by supplying animals from all three strains. Since successful transplantation studies depend upon the use of genetically defined strains of mice the cause of the failure thus became obvious. Even minor differences among strains may be responsible for inability of transplants to survive.

The seventh transplantation experiment was performed with each of the three varieties of donors which had received 0.05 ml packed human red blood cells on January 2, 1960. The animals were decapitated on January 28, 1960, spleens removed, minced with a knife, washed and the equivalent of 1/4 of an organ injected into the homologous recipient. Animals were bled 4, 7, 12, 19, 32, and 46 days after transplantation. As shown on Table 15, antibody

TABLE 15

January 28, 1960 Transplantation

SALINE

Strain	Days after transplantation 4	7	12	19	32	46
NIH	8	8	16	16	8	128
Inbred	0	0	0	0	0	0
Webster	0	0	0	0	0	0

ALBUMIN

Strain	4	7	12	19	32	46
NIH	32	16	64	128	16	256
Inbred	0	0	0	0	0	0
Webster	0	0	0	0	0	0

COOMBS

Strain	4	7	12	19	32	46
NIH	32	8	64	16	16	128
Inbred	0	0	0	0	0	0
Webster	0	0	0	0	0	0

NIH - National Institute of Health original Webster strain
Inbred - Webster inbred strain
Webster - Webster strain regular

was detectable in the NIH strain for as long as 46 days after transplantation. Homologous transplanted cells of the Inbred or Webster strains did not form antibody.

Additional experiments are being conducted to determine whether or not the original results of Frisch and Davies can now be obtained with the NIH strain of mice.

SUMMARY

The data show that the two sorts of antibody behave similarly *in vitro* and *in vivo*. Their separation was not achieved by heating the serum to various temperatures, by allowing serum to remain in contact with the clot for periods of time before titrations, by varying incubation temperature, except for a slight decrease in saline antibody at 5°C.

Single injections of antigen in varying doses, low and high protein diets, extremes of environmental temperatures and the replacement of intact cells by stroma alone or mixed with hemoglobin did not alter the incomplete - complete antibody ratio.

As a general rule the ratio of incomplete - complete antibody increased significantly when animals received multiple injections over a long period of time.

It was hoped that transplantation from donor to recipients would assist in the isolation of incomplete antibody cells. These however were not successful because of strain variations which were introduced at the mouse farm.

Experiments demonstrated that the antibody of the complete and incomplete type is produced to the stroma rather than the hemoglobin. In addition normal mouse serum was shown to contain a naturally occurring "incomplete" antibody for human red cells.

The specificity of this antibody was not related to common blood types; it was reactive against 76% of the total cells tested in dilutions of 1:20.

Pooled normal mouse serumwas also shown to agglutinate rabbit, rat and hamster cells but not cow, dog, guinea pig, goat, sheep, frog or iguana.

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