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THE INDIVIDUAL SPECIFICITY OF DOG PLASMA

by

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PREFACE

This work arose from the chance observation of a phenomenon which had received almost no attention in the literature. The problem, because it was a new one, required exploration before the definitive experiment could be designed. The results described here are from these exploratory experiments; they do not explain the underlying mechanism of the phenomenon, and indeed they pose more questions than they answer. However, the results do make it easy to defend the thesis that dog plasma has an individual specificity, and they also emphasize the physiological and clinical significance of this phenomenon.

The first two chapters are reviews of the literature, and contain none of my own experimental work. It has been helpful in these introductory chapters to range rather far afield in order to

put the thesis in proper perspective. I have paid particular attention to the phenomenon of homograft rejection, not so much because of the possibility that the specificity of plasma is directly related to it, but because both phenomena are akin in representing unusual degrees of individual specificity, and because the one phenomenon has become, and the other may become, a meeting point of genetics, immunology, physiology and clinical medicine.

Much of the experimental work described here has already been published.* I have quoted freely, where appropriate, from the parts of these papers which represent my own writing.

* with Stewart, 1957; with Stewart and Fuller, 1958; with Johns and Burgen, 1959; with Walker, 1959.

CHAPTER I

INDIVIDUAL SPECIFICITY AMONG BIOLOGICAL MATERIALS

(A REVIEW)

In some species, two materials - living nucleated cells and erythrocytes - possess a sufficient degree of specificity to enable one, by appropriate means, to use them to distinguish one individual from all or nearly all of the rest of the population. One purpose of this chapter is to assess these two examples of individual specificity. The other purpose is to show that plasma and serum also may possess qualities such that one can identify a particular plasma or serum with a particular animal.

No mention is made in this chapter of such parameters as body size, oxygen consumption, nitrogen excretion, and the like, any one of which shows variation from one animal to another, nor are the individually specific morphological characteristics, best represented by fingerprints, discussed. The only justification for this exclusion, aside from the limitations of space and time, is that these latter items do not seem quite so pertinent to the problem at hand.

A. Transplantation Immunity

Tissue transplanted from one region of the body to another - an autograft - generally survives indefinitely provided proper physiological and surgical principles are observed in the grafting. In contrast, tissue transplanted with the same care from one individual to another of the same species - a homograft - survives for only a

few days and then is rejected by the host. This phenomenon of rejection, known as transplantation immunity, is almost universal among the higher animals, and it represents the most refined example of individual specificity.

The genetic basis. It is generally accepted that the individual specificity of transplantation immunity is a result of genetic differences between individuals. Barnes and Krohn (1957) have summarized the reasons for believing that the survival of a homograft is governed by the presence or absence in the host of 'histocompatibility' genes present in the donor. That is, if the host possesses all the histocompatibility genes which are present in the donor, the graft will be accepted; if the host does not, the graft will be rejected. The presence of genes in the host which are absent in the donor does not affect the survival of the graft. Using an ingenious system of interbreeding, Barnes and Krohn have shown that there are a minimum of 15 such genes controlling the fate of grafts from one particular inbred strain of mice to another. Because the strains were genetically homogeneous, a minimum of 15 loci must be involved in histocompatibility between these two strains.

The most extensive studies of histocompatibility genes have been done by Snell in the United States and by Gorer in England. Their work has been concisely summarized by Allen (1955). Snell in particular has concentrated on one locus (locus H-2) controlling histocompatibility in mice, and has found at least ten alleles at this locus. If the other histocompatibility loci have as many alleles, and if each allele is active, then, using Barnes and Krohn's figure for the number of loci,

one can calculate that the probability of success of a graft between two mice chosen at random from the wild (non-inbred) population is of the order of $(\frac{1}{10C_2})^{15}$ or $(\frac{1}{45})^{15}$. This figure assumes complete heterozygosity, and random distribution of alleles, both of which are unlikely, and there is no solid evidence for some of the other assumptions involved in its calculation. However, it does serve to show how a genetic basis of transplantation immunity can confer upon a tissue complete individual specificity.

The immunological mechanism. The temporary survival of a homograft, and its subsequent rapid destruction, bear a suspicious resemblance to an immunological phenomenon, with the survival period representing the sensitizing interval, and the destruction the appearance of effective amounts of antibody. Only recently, however, has it been possible to translate this superficial resemblance into specific immunological terms.

The first clear evidence pointing to an immunological basis of homograft rejection was provided by Medawar (1944) who described the now familiar 'second-set' phenomenon. He showed that an animal which has already rejected one graft rejects a second graft from the same donor much more rapidly. Medawar's interpretation of this phenomenon is that the first graft sensitizes the host, and makes it immune in the classical sense, so that subsequent grafts are met with a forewarned or already immune host.

The next important advance in the immunological interpretation was made by Mitchison (1953), who showed that passive transfer of the second-set type of transplantation immunity can be effected, provided that living cells rather than serum are transferred. The tissues of the primary host most efficient in transferring immunity are the lymph

nodes draining the immunizing graft's site. Mitchison worked with tumours; Billingham, Brent and Medawar (1955) have shown that the same principles hold true in the transplantation of normal tissues.

The second-set phenomenon, the passive transfer of homograft immunity, and the demonstration that transplantation immunity can be abrogated according to the principles of immunological tolerance (as discussed later), have led most workers to accept the immunological interpretation of transplantation immunity, i.e. that homografts are antigenic and owe their destruction to the formation of antibodies against them. However, there is still no general agreement either about the nature of the antibodies or about the mechanism by which they destroy grafts. The observation that only living cells and not serum are effective in transferring immunity argues against the antibodies being of the classical type. Considerable support has been lent this idea by the demonstration by Weaver, Algire and Prehn (1955) that grafts survive indefinitely if they are placed in diffusion chambers which allow free access to the graft of host's interstitial fluid but not of host's cells. If the usual type of humoral antibody were the sole agent responsible for rejection, the grafts should not survive. It has been shown repeatedly, however, (e.g. Amos, Gorer, Mikulska, Billingham, and Sparrow, 1954) that antibodies do appear in the host's serum after homografting. Although these antibodies are agglutinins of donor's red cells and leucocytes, their occurrence after homografting may have little or nothing to do with the destruction of the graft. In fact, their appearance may be simply a response to erythrocyte agglutinogens shared by the grafted cells (that at least some red cell agglutinogens are present in other types of cells has

been shown by Coombs, Bedford, and Rouillard, 1956). Nevertheless, Gorer (1958) believes that these antibodies may be cytotoxic in vivo if not in vitro, and that the failure of passive transfer with serum may be peculiar to mice, the species used in earlier studies. Stetson and Demopoulos (1958), working with rabbits, claim to have passively transferred homograft immunity with serum after unusually vigorous immunization with homologous spleen cells and adjuvants. Most workers, however, are reluctant to accept the orthodox nature of homograft antibodies, and prefer to agree with Medawar and his colleagues (Brent, Brown and Medawar, 1958) that the rejection phenomenon requires the intercession of living cells.

The antigens involved in transplantation immunity are less controversial, not because more is known about them, but because only recently have they been studied at all. The first workers to evoke a state of second-set immunity to homologous tissue with non-living material were Billingham, Brent and Medawar (1956a). They disintegrated mouse spleen cells by ultrasonic radiation, and found that a water soluble component was released which was able to elicit immunity in mice such that the first actual homograft (from the donor of the spleen cells) was rejected rapidly, as if it were a second-set graft. The antigenic material could be obtained from cell nuclei but not from cytoplasm. Its solubility characteristics and susceptibility to desoxyribonuclease suggested that it was a desoxyribonucleoprotein. Further evidence that this nuclear material represents the homograft antigens has been obtained by Brent, Brown and Medawar (1958), who have demonstrated a delayed type of hypersensitivity in the skin after injection of this material into guinea-pigs made sensitive by earlier

homografting. The antigenic extract was prepared from the donor of the homograft. Berrian and Brent (1958) have also shown that the ability of such extracts to evoke homograft immunity is impaired by incubation *in vitro* with cells from an animal (from the same inbred strain as the extract treated animal) which has previously received a homograft from the donor of the antigenic material.

The importance of these observations on non-living antigenic material can hardly be overestimated, for, as Medawar (1957) points out, they provide a strong indication that cells constantly release into their environment substances intimately associated if not identical with their genetic apparatus. That this release is a *physiological* process, and not limited to homografts, is strongly suggested by an elegant experiment of Billingham, Brent and Medawar (1956b). They used the phenomenon of immunological tolerance to make mice permanently accept skin homografts. After these grafts had been alive and well in their new sites for more than a hundred days, the hosts were injected with normal (not immune) lymphoid cells from mice which were not tolerant to the same type of graft. (These mice were from the same inbred strain as the tolerant hosts; their lymphoid cells therefore did not act as homografts themselves.) The grafts in the tolerant animals underwent typical rejection. The only explanation for this outcome is that the transferred lymphoid cells were the effectors of the rejection. They therefore must have been exposed to antigenic material from the grafts, indicating that these grafts were discharging this material while still behaving as if they were autografts.

Immunological tolerance. Probably the most important single development

in the study of transplantation immunity has been the formulation of the principle of immunological tolerance. This principle owes its demonstration to transplantation studies, and transplantation studies have profited immensely by its application.

In 1945, Owen made the unusual observation that dizygotic (fraternal) twins in cattle have identical erythrocyte types far more frequently than can be accounted for by simple inheritance. He found in addition that in these cases each member of the pair actually had a mixture of two distinct types of erythrocytes. He postulated that the situation came about by an interchange of embryonal cells between the twins through vascular anastomoses during embryonic life. This finding had far reaching implications, for it suggested that an animal might tolerate foreign cells permanently if it were exposed to them sufficiently early in life. Billingham, Brent and Medawar (1953) showed that this is in fact true, and that animals which have received injections of living homologous tissue during embryonic life will permanently accept homografts of that tissue during adult life. Billingham and Brent (1956) have improved the experiments by showing that intravenous injection of foreign cells into newly-hatched chicks or newborn mice is sufficient to induce tolerance to subsequent homografts from the same source. Evidence is accumulating (see e.g. Cinader and Dubert, 1956; Cinader and Pearce, 1958) that tolerance can be similarly induced to protein antigens as well as to tissue cells. In the systems studied so far, the specificity of the induced tolerance is comparable to the specificity of antigen-antibody systems in general: the acquired tolerance is restricted to the antigen used to induce it.

The principle of immunological tolerance which has evolved from these experiments can be stated succinctly: an animal exposed to sufficient amounts of antigenic material during its early life permanently loses the ability to react immunologically against it. This principle has both practical and theoretical significance. Since it provides a method whereby animals can be induced to accept homografts permanently, it is being applied extensively and to great advantage in studies of transplantation immunity. An important application has already been cited (Billingham, Brent and Medawar, 1956b). More important, the phenomenon of immunological tolerance has provided a clue to the unknown mechanism whereby an animal can recognize, and take action against, a myriad of substances foreign to itself. It shows not only that this mechanism is a relatively late acquisition of the animal, but that the mechanism's operation can be impaired selectively and permanently.

B. Erythrocyte Antigens

Differences among homologous bloods have been demonstrated in many species, and in some species have enabled the formulation of elaborate systems of blood types or groups. These differences are usually a property of the erythrocyte, and are due to the presence on or in the erythrocyte of antigens. The antigenic pattern of a particular animal's erythrocytes is under genetic control; however, the genes governing the red cell antigens often are not randomly distributed through a population. Some genes may have such high frequencies as to make their antigens almost universal in a species; other genes and their corresponding antigens may be extremely rare. Antibodies to the erythrocyte antigens generally are demonstrated by

their ability to agglutinate or to lyse the cells bearing the antigens. The antibodies may be of the 'natural' type, occurring without known antigenic stimulus, or of the 'immune' type, requiring antigenic stimulus for formation.

These elementary aspects of erythrocyte types may be illustrated by reference to the human blood groups. (Unless otherwise noted, the information here is taken from the extensive review by Race and Sanger, 1958). So far, 57 antigens have been demonstrated on human erythrocytes, and the existence of at least 20 more is suspected. Some are so rare that they have been designated 'private' antigens, implying that they exist only in the members of one or two families. Others are so common that they have been called 'public' antigens, implying that almost everyone possesses them. The majority of antigens in man have intermediate, although still not random frequencies. For example, 92% of Englishmen possess the Lu^a antigen of the Lutheran system; 8% lack it. Although the number of erythrocyte antigens identified in man is sufficient to determine several million phenotypes, the distribution of these antigens is such that they do not confer an absolute degree of individual specificity upon erythrocytes. Using Fisher's method (1951) it can be calculated that, with the existing typing procedures, the probability of failure to distinguish between two samples of blood taken at random from the English population is 0.00042. The antibodies in man may be either natural or immune. For example, Anti-A and anti-B are almost invariably present in blood lacking the corresponding antigen; anti-Lu^a occurs after immunization but sometimes is present naturally; anti-K (Kell system) has been demonstrated only in subjects exposed to K antigen.

Although the most extensive work has been done in man, blood groups in animals have received considerable attention. Ferguson (1955) has reviewed the subject thoroughly, and describes blood group systems in cattle, horses, swine, sheep, dogs, birds, and in the small laboratory animals. Most of these systems are relatively simple and do not allow the approach to individual specificity exemplified by the systems in man. An exception is seen in cattle, however, for the use of immune sera has demonstrated more than 40 distinct antigenic factors controlled by multiple alleles at at least 10 loci.

Particular mention should be made of blood groups in dogs. The infrequency of typical hemolytic transfusion reactions in dogs has lead many people to believe that blood groups are absent in this species. As early as 1910, however, von Dungern and Hirschfeld described isoagglutinins of canine erythrocytes, and since their work there have been scattered reports, summarized by Cohen and Fuller (1953) and Hamilton (1948), dealing with this subject. Definitive work on canine erythrocyte types has been done by Young and his associates (Young, Ervin and Yuile, 1949; Christian, Ervin and Young, 1951; Young, O'Brien, Swisher, Miller and Yuile, 1952). Using isoimmune sera, and agglutination techniques, they have demonstrated 5 distinct antigens which they have designated A, B, C, D and E (no connection with human types represented by these symbols is intended). Only the A antigen was able to evoke antibody production of a type which would result in severe (hemolytic) reactions on subsequent transfusion. Naturally-occurring isoantibodies could be detected in only 15% of dogs, and usually only in low titre. They were mostly of anti-D specificity. Since only about one-third of dogs were found to be D-positive, one concludes that only about 5% of random pairs of mongrel dogs should

show an incompatibility on direct cross-matching.

An important application of this work has been the production in new-born pups of a syndrome resembling erythroblastosis foetalis (Young, Christian, Ervin, Davis, O'Brien, Swisher and Yuile, 1951). A-negative bitches, deliberately immunized with A-positive cells, were mated to A-positive dogs. The newborn A-positive pups regularly developed a fatal hemolytic disease, provided they were allowed to suckle the immunized mother during the first day of life. This observation is of particular interest in this thesis because it shows that canine maternal antibodies can be transferred to offspring, and that this transfer may occur by other than the transplacental route.

C. Plasma and Serum

The existence of interspecies variations in plasma and serum is an accepted fact, and indeed is one of the cornerstones of immunology. Homologous plasma and serum, however, have usually been considered to be rather homogeneous substances showing little if any qualitative variation among different members of the species. This opinion undoubtedly resulted from the (justified) preoccupation of most investigators with differences in the cellular components of homologous blood, and from the relatively late advent of the sophisticated techniques of the protein chemist and the immunochemist.

There is ample evidence now that qualitative differences do occur among homologous sera in some species. The purpose here is to summarize this evidence and to show that at least the potentiality exists for individual specificity of plasma and serum.

Immunological differences. One method of detecting differences in the large molecules of sera is to analyse the antigenic components

of individual sera. In 1943, Cumley and Irwin used this method to demonstrate a considerable degree of individual specificity of human serum. They induced formation in rabbits of precipitins to individual samples of human serum, and then showed that absorption of these precipitins by human serum other than that used for induction usually did not remove all the precipitins capable of reacting with the inducing serum, or indeed with some other samples of serum. After a large number of tests, all the results of which were not reported, they concluded that 'human serum contains antigenic components by which the serum of an individual can be distinguished from that of others.' Goldberg and de Gara (1948) claimed to be unable to confirm these results, but their study was on too small a scale (only two human sera were used) to be conclusive.

More recently, Oudin (1956a,b) and Dray and Young (1958) have used a modification of this method to demonstrate differences in the antigenic components of sera of individual rabbits. The latter authors had particular success; they were able to divide 90 rabbit sera into 30 groups according to their reaction patterns in agar gel with 6 antisera. The most interesting aspect of the work of Oudin and of Dray and Young is that the antisera were homologous or isoimmune, indicating that the antigens involved are antigenic in some or all of the other members of the species lacking them.

Turning to the other immunological component of serum, the antibodies, it is possible that animals may have individually specific antibody patterns in their sera as well. It is entirely possible, in fact, that the 'antigenic' differences just described may really represent differences in various antibody components able to act as isoantigens.

Only in the case of the erythrocyte isoagglutinins, however, have clear qualitative differences in antibody patterns been demonstrated regularly. Because of their limited distribution, the natural isoagglutinins do not afford much degree of individual specificity. In man, for example, only the agglutinins associated with the ABO and P systems are consistently present in the absence of known antigenic exposure (Race and Sanger, 1958). Theoretically, of course, it should be possible with appropriate immunizing procedures to provide a given serum with an isoagglutinin pattern almost as individually specific as the corresponding agglutinogen pattern. This latter example of individual specificity, however, would have to be regarded as a rather artificial system not present under normal conditions.

Physico-chemical differences. Although there had been several reports of intraspecies variations in the physico-chemical properties of the serum protein fractions (e.g. Bernfeld, Donahue and Homburger, 1953) and even a suggestion that these might be under genetic control (Thompson, Foster, Gowen and Tauber, 1954), it remained for Smithies and his associates to attempt a careful analysis of the problem. Using one-dimensional and two-dimensional starch-gel electrophoresis, which in their hands produces remarkable resolution of serum protein fractions, they have been able to demonstrate in human serum two haptoglobins (alpha$_2$-globulins) whose presence is governed by two allelic genes showing no dominance (Smithies, 1955; Smithies and Walker, 1956), and three beta-globulin fractions governed by three allelic genes without dominance (Hirsfall and Smithies, 1958; Smithies, 1958). Although the 'serum groups' elucidated by Smithies and his colleagues do not confer individual specificity on serum, they do show that this is theoretically

possible and that at least some serum protein fractions are genetically controlled. That this phenomenon is not peculiar to man is shown by the work of Ashton (1958) and Ashton and McDougall (1958), who have extended Smithies' work to other species and have found similar genetically controlled serum protein polymorphism in cattle, sheep, goats and horses.

Immediate biological differences. By this term is implied differences which enable one to distinguish between two samples of plasma or serum on the basis of the responses they produce on initial injection into an animal.

It is convenient to mention first that one can distinguish between heterologous and homologous plasma on this basis. This was best demonstrated by Lake, Simmonds and Steinbeck (1953a,b), who showed that the injection of heterologous plasma into the skin, or pleural or peritoneal cavities of non-sensitized animals produces an immediate local increase in capillary permeability with exudation. Recipient animals included rats, rabbits and guinea-pigs; a variety of heterologous donor sera were tested. The authors could not explain the phenomenon, although they showed that the protein moiety of the plasma was responsible for the effect. Antihistamines had some inhibitory effect on the reaction to intracutaneous injection, but did not affect the reaction in the serous cavities. It is possible that these immediate effects of heterologous plasma are due to a release of endogenous histamine, for, as will be described later, Feldberg and Schachter (1952) and Schachter and Talesnik (1952) have shown that histamine is released by an initial injection of horse serum in the cat, and by an initial injection of egg white in the cat and rat.

Evidence that homologous plasma and serum samples also can be distinguished according to their effects in vivo is not easy to find in the literature. It is well established, of course, that injection of homologous plasma containing appropriate isoagglutinins can produce profound reactions; however, other effects of plasma or serum, unrelated to their agglutinin content, have only occasionally been described. What reports there are deal almost entirely with the effects of intracutaneous injection. In 1927, Chant and Gay reported as an incidental observation that homologous serum often produced larger skin reactions than did autologous serum in man. (In consulting their paper, one should note that they use 'heterologous' and 'homologous' to mean 'homologous' and 'autologous' respectively). Levine and State (1942; State and Levine, 1942-3) observed triple responses to intracutaneously injected homologous plasma in about 20% of human subjects, although they do not mention the use of autologous plasma controls in their study. They were inclined to attribute the majority of the reactions to the presence of dissolved incompatible erythrocyte antigens in the plasma. Finally, Mumsell (1944) has reported frequent reactions to intracutaneous injection of homologous serum in man. Autologous serum controls did not produce these reactions, nor could she find any correlation with the ABO blood group system.

The dog is apparently the only other species in which this kind of intraspecies variation has been noted, and even here there are only two short communications by Freeman and Shecter (1942a,b) dealing with the subject. These workers, while looking for a capillary permeability factor in the lymph from injured limbs of dogs, noted that some samples did cause an increase in capillary permeability as evidenced by

the appearance of the dye T-1824, previously given intravenously, at the sites of intracutaneous injection of such samples. The unusual aspect of this effect was that it occurred only if the samples were tested on another dog. Plasma and serum obtained from normal animals were then tested in the same way; it was found that they regularly produced local increases in capillary permeability when injected intracutaneously into other dogs, but not when given to the dog from which they were obtained. All combinations were tested in 6 mongrel dogs and no exception was found to the rule. The authors concluded that in their limited experience, dog plasma and serum possess an individual specificity.

CHAPTER II

SOME ASPECTS OF THE RELEASE OF HISTAMINE

(A REVIEW)

Since some, at least, of the immediate effects of foreign protein injection can be attributed to the release of histamine in the recipient, it may be as well to examine the physiological disposition of histamine in the tissues and the conditions under which it can be released in a free and active form.

A. The State of Histamine in the Body

Histamine is a normal constituent of most mammalian tissues. Very little, if any, however, is present in a free form under normal conditions. Until recently, the exact site where histamine is held in the tissues was unknown. From the observations of Riley and West (1953) it is now clear that the greater part of the histamine in most tissues is contained in the mast cells. These workers showed a close correlation between histamine content and mast cell content, and a selective damage to mast cells by compounds known to release histamine in the body. Their postulate that mast cells are rich in histamine has been amply confirmed, particularly by the demonstration by Benditt, Wong, Arase and Roeper (1955) and Garcia-Arocha (1958) of histamine release from mast cells isolated by the technique of Padawer and Gordon (1955). Histamine therefore joins heparin (Jorpes, 1946) as a normal constituent of the mast cell.

It should not be implied that because mast cells are rich in histamine all the body histamine is in the mast cells. None of the

methods used to show histamine in mast cells has been sensitive enough to show that all a tissue's histamine is in its mast cells. Moreover, apparently much of the histamine in the gastrointestinal tract is not in mast cells (Mota, Beraldo, Ferri and Junqueira, 1956), although its true site is unknown. This point is mentioned to show the possible error in assuming a substance to be a mast cell depleter on the evidence that it releases histamine.

Another pitfall relevant to the association of histamine with mast cells is to suppose that an effect which is suspected to be due to histamine release is in fact due to this cause because it is absent in tissues depleted of their mast cells. Not only do mast cells contain heparin along with histamine, but also at least one other substance with potent pharmacological properties, 5-hydroxytryptamine (Benditt, Wong, Arase and Roeper, 1955). Although the distribution of 5-hydroxytryptamine is not so universal among mast cells (Parratt and West, 1957a; Cass, Marshall and Riley, 1958), the similarity of some of its pharmacological actions with those of histamine warrant its careful assessment in effects suspected of being histamine mediated.

B. Methods for Detecting Histamine Release

Detection of free histamine. Real proof of histamine liberation in a given phenomenon ultimately must rest on a demonstration of the appearance of free histamine.

Detection of increased levels of histamine in the blood plasma would appear at first glance to be the easiest way to show histamine release. That this is not always the case can be attested to by anyone who has tried it. Released histamine, owing to its high diffusibility and aqueous solubility, rapidly undergoes enormous dilution, not only by

the plasma but by the entire extracellular fluid. The assay of histamine in plasma may be interfered with by the presence of other agents. Rough handling, hemolysis or clotting of blood samples raises the plasma histamine content (Emmelin, 1945). These various difficulties have been partially overcome, however, by using the venous or lymphatic effluent from histamine-rich tissues rather than random venous samples (e.g. Dragstedt and Gebauer-Fuelnegg, 1932; Gebauer-Fuelnegg and Dragstedt, 1932), by extracting the histamine from plasma (reviewed by Code, 1952) and by handling blood samples gently (Emmelin, 1945).

The annoyances in detecting free histamine in blood have led to the substitution of isolated preparations for whole animals. Generally, such preparations have the advantages of allowing the selection of material particularly rich in histamine, of reducing the dilution of freed histamine, and of permitting perfusion or incubation with media free from interfering substances. Among the more widely used preparations (Mongar, 1956) are perfused skin, chopped lung, rabbit platelets, and isolated intracellular particles.

Detection of histamine depletion. Demonstration that the histamine content of a preparation is reduced by a given treatment gives presumptive evidence of histamine liberation. For example, Ojers, Holmes and Dragstedt (1941), by assaying samples of liver before and after anaphylactic shock, were able to show that large amounts of histamine were released from this organ during anaphylaxis in the dog. The method is a relatively simple one, but unless the reduction in histamine is large, it may require many experiments, for the histamine content often shows wide variations, not only in different animals and tissues, but in different regions of the same tissue (see e.g. Feldberg and Miles, 1953).

Detection of effects of free histamine. Because histamine has such a variety of pharmacological effects, evidence for its release can often be obtained by observing these effects. Thus arterial hypotension, increased hematocrit, increased capillary permeability, smooth muscle contraction, urticaria, oedema, and gastric acid secretion are among the responses which have been cited as evidence for histamine release.

As a rule, the occurrence of one or more of these responses is used only as it should be, viz, as suggestive or circumstantial evidence. This rule does not do justice, however, to two types of response which are particularly useful in incriminating histamine: increased gastric acid secretion, and what can be called the species-specific response to histamine.

Gastric acid secretion as an index of histamine release has several merits. It is extremely sensitive; intact dogs will respond to as little as 0.02 μg./kg./min. given intravenously, and human subjects to 1/10th this dose (Hanson, Grossman and Ivy, 1948). More important, it is unusually reliable. Histamine is not unique in stimulating gastric secretion, but in most experiments in which its release is suspected, the action of other gastric secretagogues is unlikely. This is particularly the case since the demonstration by Black, Fisher and Smith (1958) that 5-hydroxytryptamine does not stimulate gastric secretion. Finally, the pattern of secretion evoked by histamine is quite characteristic, the juice being highly acid, copious, and low in pepsin (Gilman and Cowgill, 1951).

The species-specific response to histamine is a consequence of the fact that in at least some species one response to histamine predominates over all others, and that this response is characteristic of

the species. To illustrate: if a guinea-pig, a rabbit, and a dog are each given a large dose of histamine, or of a substance able to release histamine, the predominant effect will be asphyxia from bronchiolar constriction in the guinea-pig, pulmonary hypertension from pulmonary arteriolar constriction in the rabbit, and portal congestion from hepatic venous constriction in the dog (Dale and Laidlaw, 1910-11; Feldberg, Schilf and Zernik, 1928; Mautner and Pick, 1929). These responses are so characteristic of the species that individually they provide a rather good indication of histamine release, and collectively almost conclusive proof.

Use of antihistamines. Inhibition of a response with an antihistamine is probably the easiest method of implicating a histamine release mechanism.

The reliability of evidence obtained with antihistamines depends largely on their specificity. It is generally agreed that the commonly used antihistamines, particularly mepyramine, have a high degree of specificity, provided they are not used in massive doses (Schild, 1947; Reuse, 1948; Bain , Broadbent and Warin, 1949). That is, if a response is inhibited by a dose which is just adequate to inhibit the response to administered histamine, the evidence is good that the response is due to histamine. The difficulty arises when relatively large doses of the antihistamine are given. Here no safe conclusions can be drawn; if the response is inhibited, the effect may be a non-specific one; if it is not inhibited, it may still be due to histamine, for, as discussed by Dale (1948), it is possible that sometimes histamine may be released in such high local concentration as to require enormous doses of antihistamines for its antagonism.

Use of histamine depletion. Whether an effect is due to a release of histamine can sometimes be decided by its presence or absence in tissues depleted of their endogenous histamine. Thus rats (Brocklehurst, Humphrey and Perry, 1955) and rabbits (Lecomte, 1956) pretreated with the histamine liberator compound 48/80 still showed Arthus reactions, and the authors concluded that histamine plays a minor role. With this type of experiment, and this kind of result, the conclusion is sound so long as it is shown that the endogenous histamine has in fact been depleted. However, in experiments where the other kind of result is obtained - i.e. where the effect is reduced - it is not always safe to conclude that histamine release is an essential factor. Pretreatment with a 'histamine liberator' usually means pretreatment with a mast cell depleter, and therefore a depletion not only of histamine, but also of heparin, 5-hydroxytryptamine and perhaps other unknown factors. For example, Feldberg and Talesnik (1953) showed that the anaphylactoid reaction to egg white in rats was reduced after prolonged treatment with 48/80 and concluded that histamine was an important mediator of the response; Parratt and West (1957), however, later showed that 5-hydroxytryptamine is much more significant in this reaction, but is also depleted by 48/80.

C. Histamine-releasing Substances

Of natural origin. These have been reviewed by Paton (1957) and Rocha e Silva (1955), and include trypsin, 5-hydroxytryptamine, leucotaxin, and some lymphagogues, venoms, and amino acids. Two substances are of particular interest - horse serum and egg white.

Feldberg and Schachter (1952), using perfused skin flaps, found that horse serum released large amounts of histamine from the

skin of non-sensitized cats. The release resembled that obtained with compound 48/80, the maximum output occurring during the first few minutes after the addition of horse serum to the perfusate. Dog and rabbit serum did not have this effect, although human serum, on the two occasions in which it was tested, released small quantities of histamine. Autologous cat serum was without effect; apparently homologous cat serum was not tested. The significance of these observations is stated by the authors: 'the finding that horse serum releases histamine from the skin of the cat without previous sensitization of the animal appears to be the first direct demonstration of the release of histamine by a foreign protein not previously activated'. Curiously, these workers were not able to show a similar phenomenon in dogs; using perfused skin, they could obtain histamine release by horse serum only in animals which were so strongly sensitized by previous injection that they reacted with anaphylactic shock to intravenous injection of horse serum.

Experiments along similar lines were done by Schachter and Talesnik (1952), using egg white instead of horse serum. They found that egg white also released histamine from perfused skin of non-sensitized cats, but not from the skin of non-sensitized dogs. They were able to show too that intravenous egg white raised the plasma histamine of rats. The active agents in egg white were different in the cat and rat; the component effective in the rat was heat stable, in the cat heat labile.

Of simple chemical structure. The classic demonstration by MacIntosh and Paton (1949) that a number of basic organic compounds of simple chemical structure liberate histamine on initial injection without

producing other obvious effects has led to the recognition of a variety of compounds known collectively as 'histamine liberators.' These substances have recently been reviewed by Paton (1957), and the only member that will be considered here is 48/80, the compound used in some of the experimental work of this thesis.

Compound 48/80 was prepared by Baltzly, Buck, deBeer and Webb (1949) from the condensation of p-methoxyphenethylmethylamine with formaldehyde. Its potent histamine-releasing action was demonstrated by Paton (1951), who showed that it caused a depressor response and a rise in plasma histamine in the cat and dog, a rise in intraportal pressure in the dog, and a triple response in human skin. Feldberg and Schachter (1952) showed that 0.1 - 0.5 mg. would release a large fraction of the histamine of a perfused flap of skin in the dog.

Although histamine liberation is the predominant effect of 48/80, this compound also releases or activates other substances in vivo. Paton (1951) found that after its administration to cats and dogs, the rise in plasma histamine was accompanied by the appearance in the plasma of a factor ('slow-contracting substance') which caused a slow contraction of the guinea-pig ileum even in the presence of mepyramine. He also noted a prolongation of the clotting time, almost certainly a result of released heparin. More recently, Bhattacharya and Lewis (1956) have shown that 48/80 is a potent liberator of 5-hydroxytryptamine in the rat. Compound 48/80 therefore may be described more accurately as a mast cell depleter.

Of immunological significance. It is well established that histamine, and in some species heparin, are released in sensitized animals exposed to antigen. Many reviews have dealt with the role of histamine in the

anaphylactic and allergic reactions, and only some interesting recent developments will be mentioned here.

Evidence is now accumulating that, just as in the case of the reaction to the histamine liberators like 48/80, pharmacologically active substances other than histamine and heparin are released in the anaphylactic reaction. Beraldo (1950) has found a bradykinin-like substance in the plasma of dogs in anaphylactic shock. Brocklehurst (1953) has shown that a 'slow-reacting substance' appears in the perfusate of lungs of sensitized guinea-pigs when antigen is added to the preparation; this substance is not 5-hydroxytryptamine or bradykinin, although it has some of the properties of the latter. With the demonstration by Parrat and West (1957b) that 5-hydroxytryptamine is the most important mediator of the anaphylactoid reaction in the rat and by Bhattacharya and Lewis (1956) that 5-hydroxytryptamine is released by 48/80, it becomes proable that 5-hydroxytryptamine is released as well during the anaphylactic reaction in at least some species. That this is in fact so in the rat has been shown by Garcia-Arocha (1958), who found that large amounts of 5-hydroxytryptamine were released from the perfused gut of sensitized rats after the addition of antigen to the perfusate. Garcia-Arocha has also been able to partially protect rats from anaphylactic shock with antagonists of 5-hydroxytryptamine.

Probably the most interesting development in the field in some years is the finding of Germuth and McKinnon (1957) that antigen-antibody complexes formed in vitro and dissolved in excess antigen produce typical anaphylactic shock when injected into normal, non-sensitized animals. McIlreath (1958) has shown that such complexes release histamine in the perfused guinea-pig lung. Ishizaka and Campbell (1958) have shown that

soluble complexes produce immediate skin reactions in normal guinea-pigs. The latter workers also noticed the effect described by Lake, Simmonds, and Steinbeck (1953a,b; Chapter I), and they make the ingenious suggestion that the skin reactions to initial injections of heterologous sera may be caused by the presence in such sera of soluble antigen-antibody complexes.

CHAPTER III

THE INDIVIDUAL SPECIFICITY OF DOG PLASMA

During some experiments involving the intrapleural injection of isotonic saline in dogs, Stewart and Burgen (1958) obtained results which could be accounted for only on the basis of increased pleural capillary permeability. It seemed likely that this change was due to the intrapleural activation of a humoral permeability-increasing factor, and we therefore attempted to demonstrate such a factor by the intracutaneous injection of samples of pleural fluid into guinea-pigs. We found from the outset that not only do some samples of pleural fluid increase capillary permeability in the guinea-pig, but also samples of fresh or stored canine plasma and serum. This result was attributed to the phenomenon described by Lake, Simmonds and Steinbeck (1953a,b) as discussed in Chapter I.

To circumvent this effect of heterologous serum protein, we used dogs as recipients for the testing of pleural fluid samples. It became apparent at once, however, that normal homologous plasma in the dog produces local increases in capillary permeability on intracutaneous injection. The effect was not observed when autologous plasma was tested. These findings indicated that canine plasma possesses at least some degree of individual specificity. Evidently we were dealing with the same phenomenon as that described by Freeman and Schecter (1942a,b; Chapter I), although at the time we were unaware of their work.

The results described in this chapter are mainly those from experiments designed to test the degree of individual specificity of dog plasma, to examine the simpler characteristics of the phenomenon,

and to determine whether it fits any accepted genetic or immunological concepts.

Some of the experiments with mongrel dogs were performed with the assistance of Dr. P. B. Stewart. Dr. J. L. Fuller provided the facilities and animals for the experiments with inbred animals.

METHODS

The skin was used as the test site. The injected plasma was always obtained from donors of the same species, in this case dogs, as the recipient; we distinguish, however, between plasma from the recipient itself, autologous plasma, and plasma from a different animal of the same species, non-autologous plasma.

Five ml. blood samples were obtained by venipuncture. Each sample was mixed with 0.1 ml. of 1% sodium heparin (Mann) in isotonic saline and the plasma separated by centrifugation at 3000 rpm for 10 min. (r = 10 cm.). Plasma samples so obtained were tested within one hour. In some experiments, three-fold serial dilutions of autologous and non-autologous plasma in isotonic saline were prepared to a final dilution of 1/81, and injected immediately. (Here, a dilution of 1/3 means plasma and saline in the proportions of 1:2, a dilution of 1/9 means plasma and saline in the proportions 1:8, etc.). Autologous and non-autologous plasma samples were always prepared and tested in the same way.

Before testing, recipient animals were 'blued' by the intravenous injection of 5 mg./kg. of T-1824 (Evans Blue, Warner-Chilcott) made up as a 1.5% solution in isotonic saline. T-1824 is known to bind securely to the plasma albumin (Rawson, 1943). When test samples were thereafter injected into the recipient's skin, the degree of dyeing

at the injection site provided an index of increased capillary permeability (Miles and Miles, 1952). Duplicate intracutaneous injections of non-autologous and autologous plasma samples were made on the clipped back of the recipient with tuberculin syringes fitted with 26 gauge needles. Injections were made within one hour of the blueing of the recipient. The volume injected was 0.1 ml. Test sites were examined 30 min. after injection; the diameter of the blued area was measured, and when this exceeded 3 mm., the intensity of blueing was graded from 1 to 4 in order of increasing intensity.

All animals were unanaesthetized.

Other procedures, particularly those relating to the experiments on young animals, are described in the text.

RESULTS

In mongrel dogs. Adult animals of both sexes, possessing their permanent teeth, were randomly selected from the stock of the Animal Colony at McGill University. There was no indication that any of these dogs had previously received any injection of biological or pharmacological materials.

Fig. I shows the responses in a typical experiment, and Table I presents the results in ten recipients chosen at random from the records. To date, samples of plasma from 105 mongrel dogs have been tested on as many recipients. When one takes into account the testing of one donor plasma on more than one recipient, and the testing of several donor plasmas on only one recipient, a total of 170 donor-recipient pairs has been examined. Intracutaneous injection of autologous plasma in these animals either had no effect, or produced a blued area which was nothing more than the traumatic mark left by the needle puncture. A few

FIGURE I - Cutaneous Responses of a Mongrel Dog.

Each square contains duplicate intracutaneous injections of 0.1 ml. D_2, E_4, and E_5: autologous plasma; E_2 and E_3: non-autologous plasma from two donors; D_3: histamine in isotonic saline, 1 µg./ml. (calc. as the base); D_4: histamine in isotonic saline, 0.1 µg./ml.; D_5: isotonic saline. Photograph taken 45 minutes after intravenous injection of T-1824, 5 mg./kg., and 30 minutes after the intracutaneous injections.

TABLE I - Cutaneous Responses of Mongrel Dogs to Autologous and Non-Autologous Plasma. Each value represents the mean from duplicate injections of 0.1 ml. All recipients and all donors were different animals.

Recipient	Autologous Plasma		Non-Autologous Plasma	
	Diameter of blued area	Intensity	Diameter of blued area	Intensity
No. 1	0 mm.	-	11.3 mm.	4
2	0	-	16.5	4
3	0	-	10.7	2
4	2.0	2	8.5	4
5	0	-	11.2	3
6	4.5	1	11.7	4
7	0	-	11.0	2
8	0	-	15.0	4
9	1.5	4	13.8	4
10	1.0	4	9.0	4
Mean			11.9	
± S.E.			±0.8	

TABLE II - Effect of Dilution on Cutaneous Responses to Non-Autologous Plasma. The results are from 50 different samples of donor plasma, each tested in a different recipient.

Highest dilution producing blueing	Number of donors	Percentage of donors
Undiluted	6	12
1/3	20	40
1/9	14	28
1/27	8	16
1/81	2	4
	50	100

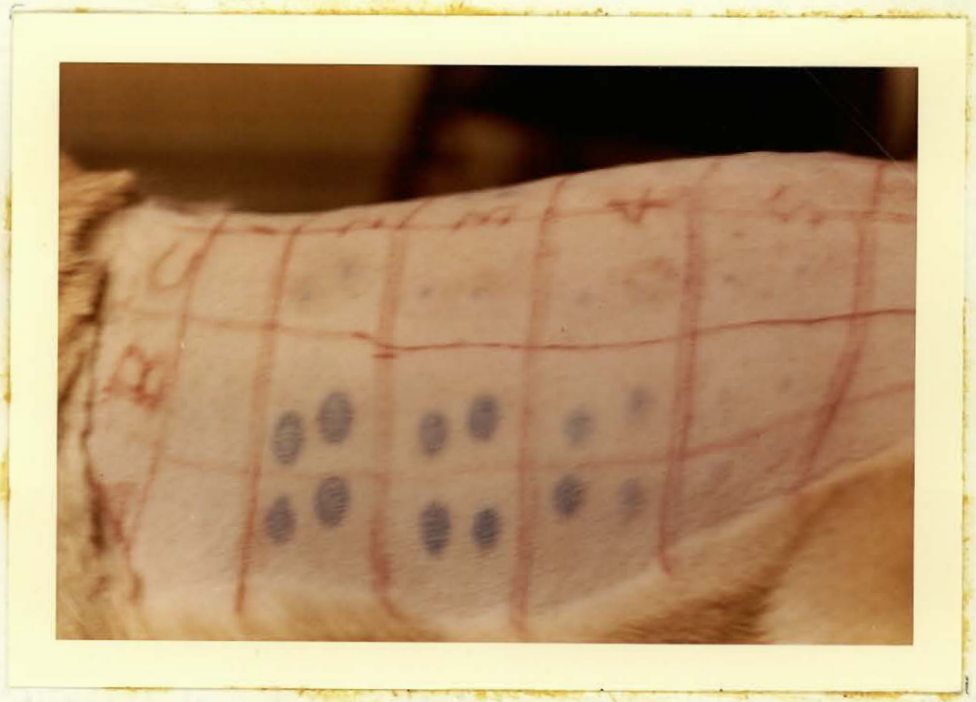


FIGURE II - Cutaneous Responses of a Mongrel Dog to Plasma Diluted with Isotonic Saline.

$C_{2,3,4,5}$: autologous plasma undiluted, diluted 1/3, diluted 1/9, diluted 1/27; $B_{2,3,4,5}$: non-autologous plasma undiluted, diluted 1/3, diluted 1/9, diluted 1/27; $A_{2,3,4,5}$: another sample of non-autologous plasma undiluted, diluted 1/3, diluted 1/9, diluted 1/27. Photograph taken 60 minutes after injection of T-1824, and 30 minutes after the intracutaneous injections. This recipient is also shown in Figure I.

animals irregularly showed a more distinct response, perhaps because the needle penetrated a region that happened to be rich in mast cells. In striking contrast was the effect of non-autologous plasma. Blueing became obvious within five minutes of injection, reached maximum size and intensity over the next 10-15 minutes, and declined slowly over 24 hours or more. Careful inspection usually showed that the bleb produced by the initial injection of non-autologous plasma had increased in size and was raised above the level of the surrounding skin.

It should be emphasized that, in every animal tested, non-autologous plasma produced a more pronounced response than autologous plasma. Occasionally, the response to non-autologous plasma was weak; when this occurred, injection of both kinds of plasma into the skin of the neck rather than the back always revealed a clear distinction between the responses to the plasmas. Why the neck area should be superior for showing the difference has not been investigated, although a greater histamine content seems to be a likely reason.

In many experiments, fresh serum was tested in the same way as plasma. It was just as effective in showing the selective response.

In order to exclude the possibility that dogs that had been housed together might have become sensitized to one another's dander, some dogs were tested within an hour of being delivered to the animal colony; their responses were no different from those of animals that had been kept for weeks.

The ability of non-autologous plasma to produce intracutaneous blueing decreased with dilution of the plasma in isotonic saline. Activity was usually retained at 1/3 or 1/9 dilutions, but was lost in the majority of tests at 1/27. A few samples retained their activity

until diluted to 1/81. The results are summarized in Table II, and the responses in one experiment are illustrated in Fig. II. Autologous plasma dilutions were always used as controls. They never produced blueing, even when diluted samples were allowed to incubate at room temperature for one hour before injection. In three experiments, autologous plasma and saline were compared as diluents of non-autologous plasma. The highest dilution producing a response was the same for both diluents in each experiment.

In a few experiments, skin testing was performed in the absence of dye. The responses to non-autologous plasma were again more pronounced than those to autologous, although the difference was not of course so striking. Careful inspection revealed that sites of non-autologous injection took on a dusky hue within one to two minutes (probably the canine version of the flare), and after five to ten minutes the wheals raised by non-autologous plasma were visually and palpably larger than the original blebs at the time of injection. These changes did not occur at sites of autologous injection. In three experiments, T-1824 was given intravenously in the usual way five minutes after the intracutaneous injection of plasma. In every case, in less than 30 seconds, the sites of non-autologous injection turned blue.

In purebred and inbred dogs. Most of the animals were from the dog colony of the Roscoe B. Jackson Memorial Laboratory. The majority of experiments were conducted on the pure-bred lines of Basenjis and Springer Spaniels whose pedigrees are shown in Fig. III. These lines had been inbred for several generations, though not long enough to produce the degree of homozygosity found in strains of inbred mice and

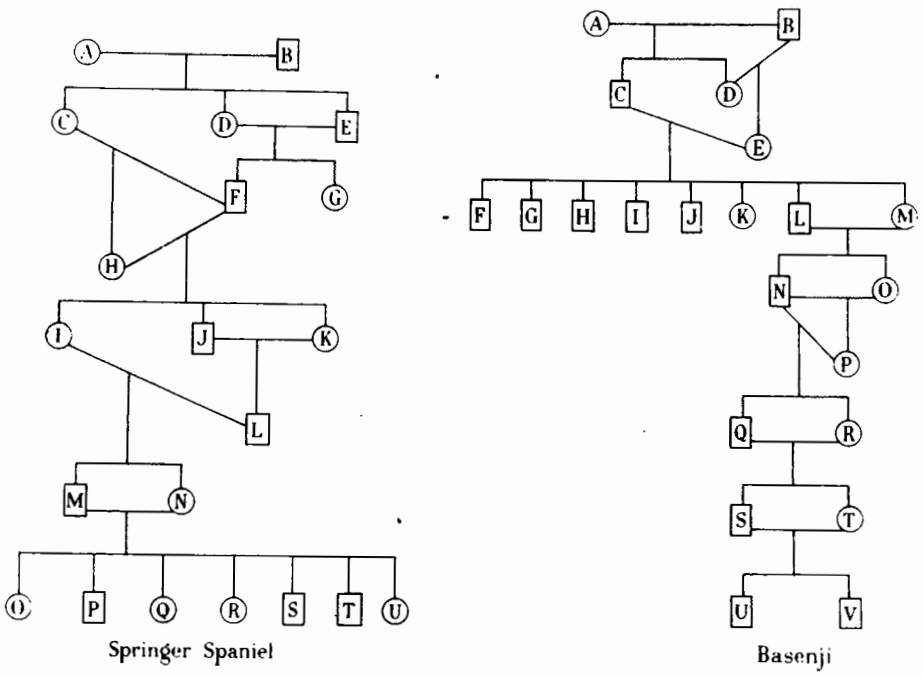


FIGURE III - Pedigrees of the Springer Spaniel and Basenji Inbred Lines.

Spaniels A and B were pure-bred siblings. Basenjis A and B were pure-bred, and partially related. Horizontal connections represent full-sib matings; diagonal connections represent back-crosses to parent or to parent's sibling. Note that neither pedigree has any outcross.

rats. All animals tested were adults. None of these animals had previously been exposed to one another's plasma, serum or tissue. All of them, however, at eight and ten weeks of age, had received anti-distemper serum prepared and pooled from the dogs of a commercial producer of veterinary products and anti-hepatitis vaccine prepared from dog liver from the same source. Some additional experiments were performed on seven pure-bred but not inbred Springer Spaniels provided in Montreal by a benefactor of McGill University. None of these latter animals had received dog serum or cellular material of any kind, or any other injection or treatment.

Results obtained with the inbred dogs are shown in Table III.

In experiment one, the responses were tested of one recipient Basenji to plasma from animals of other breeds, and to plasma from animals of his own breed. It was found that the non-autologous plasma effect occurred in these pure-bred animals, that it was pronounced, and that it did not appear to diminish as the genetic relationship between donor and recipient became closer.

Closely related Basenjis were also examined in experiment two, and the results were similar to those of the first experiment. In experiment two, 3-fold serial dilutions of plasma were also tested; this method provided a better index of plasma activity than did simple testing of undiluted plasma. The highest dilution which was still active was 1/27 for every non-autologous plasma, indicating that they all possessed approximately the same activity. Control testing of diluted autologous plasma showed no activity. In experiment three, all possible donor-recipient pairs were tested among five Basenji litter mates (animals F, G, H, I and J of the pedigree). The non-autologous

TABLE III - Cutaneous Responses of Inbred Dogs to Autologous and Non-Autologous Plasma. Each value represents the mean from duplicate injections of 0.1 ml.

Expt. No.	Recipient	Donor	Response Diameter	Intensity
1	Basenji S	Self	0 mm.	
	"	Beagle	13.0	3
	"	Cocker	11.5	4
	"	Springer	14.5	4
	"	Basenji C	12.3	4
	"	Basenji E	13.5	4
	"	Basenji K	12.0	4
	"	Basenji Q	12.8	4
2	Basenji U	Self	0 mm.	
	"	Basenji C	11.0	4
	"	Basenji Q	10.8	3
	"	Basenji V	12.8	4
	Basenji V	Self	0	
	"	Basenji U	13.8	4
3	Basenji F	Self	0 mm.	
	"	Basenji G	13.0	4
	"	Basenji H	12.8	4
	"	Basenji I	14.0	4
	"	Basenji J	13.0	4
4	Springer O	Self	0 mm.	
	"	Springer P	11.8	3
	"	Springer Q	7.0	1
	"	Springer R	11.8	3
	"	Springer S	12.0	2
	"	Springer T	9.8	2
	"	Springer U	9.5	2
	"	Springer M	12.0	3
	"	Springer L	13.0	4
	Springer Q	Self	0	
	"	Springer O	9.8	1
	"	Springer R	10.3	3

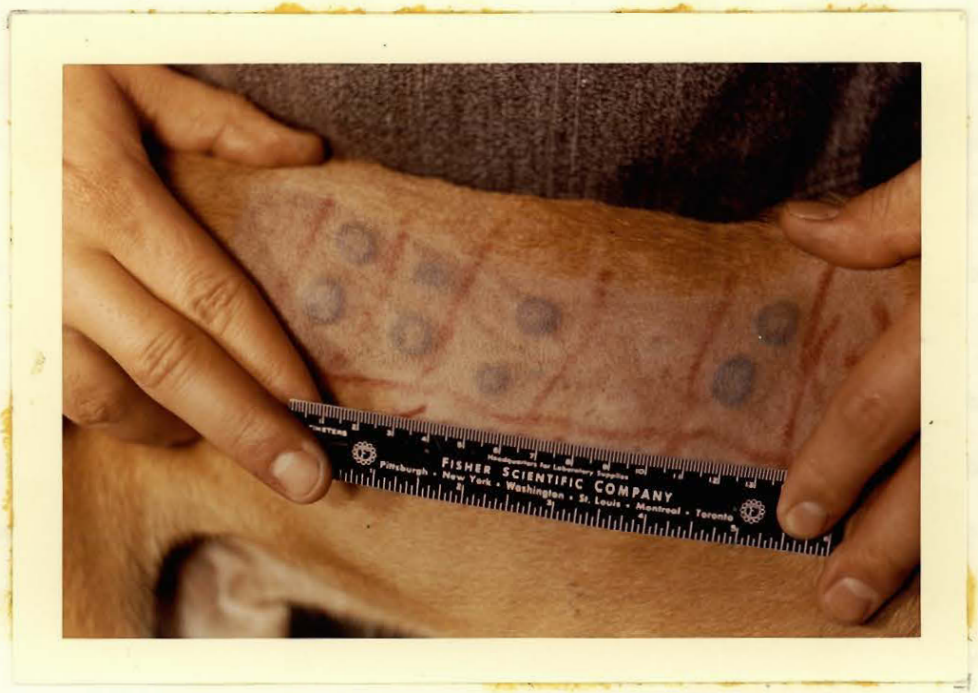


FIGURE IV - Cutaneous Responses of Basenji F.

The four squares with positive reactions contain duplicate intracutaneous injections of plasma from the recipient's four litter mates. The second square from the right contains duplicate injections of autologous plasma. Photograph taken 45 minutes after injection of T-1824 and 30 minutes after the intracutaneous injections.

plasma effect occurred in every combination. The results with Basenji F are shown in Table III, and in Figure IV; when his siblings were used as recipients, they showed just as pronounced reactions.

In experiment four, the litter mates and sires from the inbred strain of Springer Spaniels were examined. Although the non-autologous plasma effect appeared to be more variable here, it was still present in every pair. Much of the variation was probably due to the difficulty in assessing the intensity of dyeing at the injection sites, for these animals had naturally dark skin.

Many of the closely related pairs were typed for erythrocyte agglutinogens, using the system developed by Young and his associates (Chapter I). There was no correlation whatever between blood group compatibility and reactivity to non-autologous plasma.

Of the seven pure-bred Springer Spaniels obtained in Montreal, five were litter mates and the others less closely related. All possible combinations were tested; that is, 20 donor-recipient pairs among litter mates, and 22 pairs among less closely related animals. Non-autologous plasma produced the typical response in every pair; autologous plasma controls had no detectable effect. Although testing of serial dilutions was not possible, owing to limitations imposed by the small areas of light-coloured skin available in these dogs, the responses to undiluted non-autologous plasma were generally as pronounced as those in the Basenjis. In the one recipient where the responses were somewhat weaker, there was still a clear difference between the effects of non-autologous and autologous plasma.

In puppies. Plasma obtained from puppies was tested in adult recipients. Both donors and recipients were mongrel animals, unselected as to breed or

sex, from the animal colony at McGill University. None of the animals had previously been exposed to dog plasma or serum.

Two litters of puppies were followed at weekly intervals from the tenth day after birth until their plasmas produced consistently positive reactions in adult recipients. For this testing of puppy plasma, one adult recipient was used per litter per week. Blood samples (3.0 ml.) were taken from each puppy by cardiac puncture. Each sample was heparinized with 0.1 ml. of 5% sodium heparin in isotonic saline, and the plasma harvested after centrifugation. Plasma samples were left unpooled. An aliquot from each sample was serially diluted with isotonic saline. The dilutions were tested immediately, and the highest dilution at which activity was retained was noted. This procedure, by comparison with adult controls, provided a rough index of non-autologous plasma activity in those cases where neat plasma produced pronounced reactions.

As controls, one adult donor animal was assigned to the first litter (litter A) and two adult donor animals to the second litter (litter B). These control animals were bled and their plasmas tested concurrently with the puppies, on the same recipients.

Undiluted puppy plasma tested on the 10th, 17th and 24th post-natal days uniformly produced either no response, or weak responses, in adult animals. From the 31st day on, however, the activity rapidly increased to the adult level. This change is shown in Fig. V. Litter A was weaned during the fourth post-natal week. Since the increase in plasma activity to the adult level began during this period, it seemed interesting to see whether weaning influenced the time course of the increase. Weaning of litter B was therefore withheld for the duration of the experiment. Evidently this did not prevent the development of

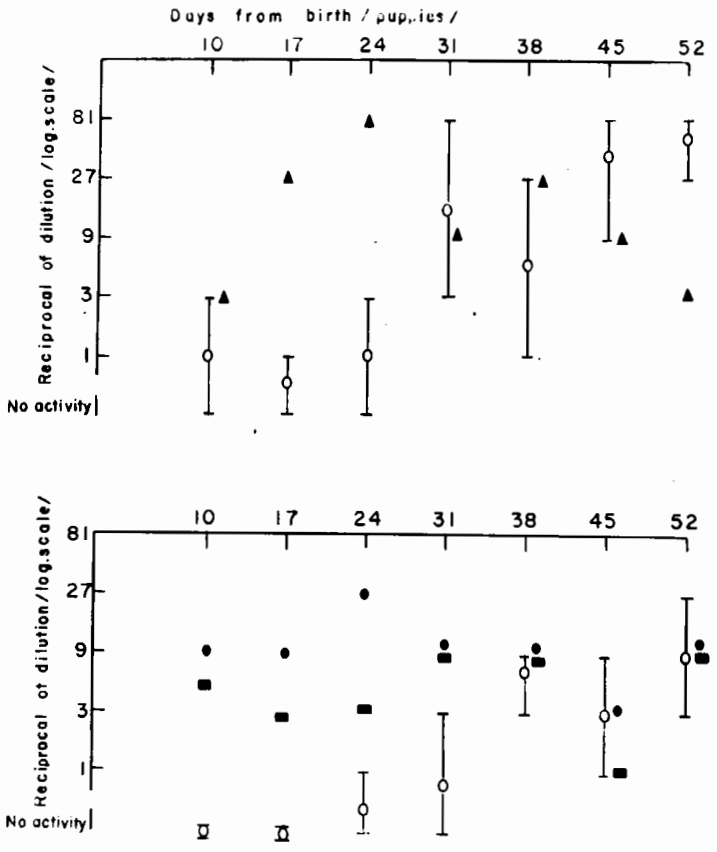


FIGURE V - Titres of Non-Autologous Activity in Plasma from Puppies and from Adult Dogs.

Litter A (above) contained 6 pups and litter B (below) 5 pups. The titre represents the highest dilution which gave a detectable response. $\bar{\varphi}$: mean titre, with full range of each litter of pups; ▲ ● ■ : titres of adult animals tested concurrently.

adult activity.

Mother-offspring relationships. In the initial experiments with puppies described above, our technique for obtaining blood by cardiac puncture was not good enough to permit the collecting of plasma for testing earlier than about the tenth post-natal day. However, as the technique improved, we were able to obtain plasma from a litter of pups twelve hours after their birth. Surprisingly, this plasma had considerable activity when tested on an adult recipient. Unfortunately the mother of these pups had very dark skin, so that skin testing in her was impractical, but it was suspected that the activity in the plasma from newborns represented transferred activity from the mother.

This idea was tested in a second litter of four pups. These pups were allowed to feedfreely at the mother. Blood was obtained at the 24th post-natal hour, and the individual samples of plasma were injected intracutaneously (a) into an unrelated adult mongrel female, and (b) into the mother. The results of this experiment are presented in Table IV. They show that the puppy plasmas were able to evoke clear responses in an unrelated adult roughly comparable to those produced by the mother's plasma, but were without effect when injected into the mother. That the mother was able to respond to non-autologous plasma was shown by her reaction to plasma from the unrelated adult. In order to determine whether the lack of response by the mother to her offsprings' plasmas represented a true tolerance to a 'non-autologous factor' manufactured by her pups or simply represented a tolerance to her own plasma factor, plasma was obtained from two of the pups when they were six weeks of age. This plasma now produced pronounced reactions in the mother, even at a dilution of 1/3.

TABLE IV - Mother-Offspring Relationships. Each reading represents the average response to at least two intracutaneous injections of 0.1 ml. of plasma.

Recipient	Donor	Response	
		Diameter	Intensity
Unrelated adult	Self	0 mm.	
	Mother of pups	8.5	4
	Pup # 1	8.5	2
	Pup # 2	9.0	3
	Pup # 3	trace	
	Pup # 4	8.5	3
Mother of pups	Self	0 mm.	
	Unrelated adult	8.5	4
	Pup # 1	0	
	Pup # 2	0	
	Pup # 3	0	
	Pup # 4	0	

A further experiment was performed on the mature offspring of several bitches at the Jackson Laboratory. Two adult Basenji siblings, and two adult wire-haired terrier siblings, were injected with their mothers' plasma. They showed clear responses to maternal plasma, responses just as pronounced as their responses to plasma from unrelated animals.

DISCUSSION

These results demonstrate clearly that canine plasma possesses a considerable degree of individual specificity. Intracutaneous injection of another (adult) dog's plasma causes a marked local increase in capillary permeability with distinct wheal formation, while a similar injection of the animal's own plasma has no such effect. The specificity is unusual and indeed seems to be unique in that it always manifests itself on the initial injection of plasma; sensitization in the usual sense is unnecessary. This is in contrast to the type of specificity exhibited by the homograft rejection phenomenon or by the majority of the erythrocyte antigens, where the specificity reveals itself only after a latent period during which antibody production occurs.

During the experiments with mongrel dogs, it seemed reasonable to attribute the specificity of dog plasma to gross ontogenetic disparities among mongrel dogs. (One need only look at our stock of mongrels to be impressed with this diversity). However, the experiments with pure-bred and inbred dogs quickly showed that this was not the case, for we have been unable to find either compatible pairs among closely inbred pure-bred animals, or even to detect any diminution in the response to non-autologous plasma as the genetic relationship between donor and recipient becomes closer.

The inbred dogs we studied had never been previously exposed to donor plasma, but it is possible, of course, that they had been in some way affected by their exposure to pooled dog serum given to protect against distemper, or by exposure to cellular material contained in the antihepatitis vaccine. It was with this in mind that we tested the pure-bred Springer Spaniels obtained in Montreal. These animals had never received injections of any kind. Since their responses were no different from those of the inbred dogs, and since they themselves were rather closely related, it seems unlikely that the early treatment of the inbred dogs need be a serious qualification to the interpretation of the present study.

The reason for testing serial dilutions of plasma was of course to see whether this procedure could be used as a rough quantitative measure of non-autologous activity, just as the titre of erythrocyte agglutinins is usually determined by dilution techniques. For the procedure to be valid, it was necessary to show, in so far as possible, that the change in non-autologous activity with dilution was in fact due to simple physical dilution, and not to a chemical activation or removal of the factor responsible for the non-autologous activity or of some other factor. The importance of this point is shown by the demonstration that simple dilution with isotonic saline of guinea-pig serum (Mackay, Miles, Schachter, and Wilhelm, 1953; Miles and Wilhelm, 1955) or of human plasma (Stewart and Bliss, 1957) activates an enzyme-like factor ('PF/Dil') which increases capillary permeability. In the dilution studies with canine plasma, therefore, autologous plasma dilutions were always used as controls. Since they never produced blueing, the responses to diluted samples of non-autologous plasma are due to the same factor

as that in undiluted non-autologous plasma. That this factor is not appreciably altered chemically by dilution in saline is suggested by the fact that use of autologous plasma instead of saline as the diluent did not alter the titre. The dilution technique therefore seems to be suitable as a rough index of non-autologous activity.

It was considered possible that the dye T-1824 might in some way be playing an integral role in the selective response to autologous and non-autologous plasma. However, none of the results of some experiments designed to test this possibility suggests that this is the case. Responses to non-autologous plasma were more pronounced than those to autologous in the absence of dye. When dye was given shortly after the intracutaneous injections, the rapidity with which the sites of non-autologous injection turned blue (little more than one circulation time) strongly suggests that the increase in capillary permeability already existed at the time of dye injection. Further evidence that T-1824 is unimportant in these responses except as a visual aid will be presented in Chapter IV.

Plasma taken from puppies during the early weeks of life is ineffective in producing the typical response to non-autologous plasma. This is particularly apparent in view of the fact that among adult dogs no instance has been encountered in which non-autologous plasma has failed to produce the expected response; among 33 samples of plasma obtained from 11 puppies during the 2nd, 3rd and 4th post-natal weeks, 20 samples failed to produce any detectable response in adult recipients, and the remainder produced only weak responses. After the 4th week, however, the activity of puppy plasma rapidly rises to the adult level. The stimulus which causes this is unknown, for although in litter A the

rise to adult level was concurrent with weaning, litter B showed a similar rise while unweaned. A rather subtle qualification which should be noted here is whether the rise to adult activity really represents the appearance of non-autologous activity, or the transient appearance of some other factor able to increase capillary permeability and absent in adult animals. It must be remembered that in the experiment as designed it was not possible to test repeatedly autologous puppy plasma, for once an animal is 'blued' it is unsuitable as a donor, and in any case skin testing in young puppies is a formidable undertaking. The possibility that the plasma activity of older puppies is not non-autologous activity seems remote, however, for we have noticed repeatedly that autologous plasma is uniformly inactive in animals without permanent teeth but sufficiently mature to permit careful skin-testing.

Although plasma taken from puppies during the early post-natal weeks is generally inactive, plasma taken during the first day of life may have considerable non-autologous activity. This suggests at once a transfer of maternal activity, and this hypothesis is strongly supported by the demonstration that while newborn puppies' plasma produces distinct responses in unrelated adult recipients, it is without effect in the mother. Furthermore, the transfer of large molecules (hemolysins) to the offspring through the colostrum has been demonstrated in dogs by Young and his associates, as discussed in Chapter I. Whether the transfer of non-autologous activity also occurs through the colostrum is not answered by our experiment, but will be tested when further litters are available.

In view of the evidence that maternal non-autologous factor apparently is present in the blood of the offspring during the early

post-natal period, it was pertinent to inquire whether the offspring might be permanently tolerant to the maternal factor owing to their early exposure to it, in accordance with the principle of immunological tolerance. When maternal plasma was tested on mature offspring, however, no tolerance to this plasma was apparent.

Possible mechanisms which might account for the individual specificity of dog plasma are discussed in Chapter VII.

CHAPTER IV

HISTAMINE RELEASE BY NON-AUTOLOGOUS PLASMA IN THE DOG

As soon as it had been established that non-autologous plasma produces a wheal on intracutaneous injection into the dog, and that this effect is not shared by autologous plasma, some simple tests were done to see whether non-autologous plasma acts directly on the skin capillaries to increase their permeability or whether it acts indirectly through one or more intermediate mechanisms. The effect of an antihistamine on the response to non-autologous plasma was tested in one of the first of these experiments; in moderate doses it produced a marked reduction in the response. This result, and the obvious similarity between the effects of non-autologous plasma and histamine, led to the hypothesis that the principal effect of non-autologous plasma is a release of endogenous histamine.

In the following experiments, four criteria for histamine release by non-autologous plasma were used: (a) partial or complete inhibition by an antihistamine of the whealing produced by non-autologous plasma, (b) reduction or absence of the whealing in skin previously depleted of its endogenous histamine by a histamine liberator, (c) an increase in gastric acid secretion after exposure to non-autologous plasma, and (d) the presence of free histamine in tissue exposed to non-autologous plasma. These criteria imply, of course, the use of suitable autologous plasma controls.

Mr. J. D. Walker assisted with the experiments testing criterion (d).

METHODS

Adult mongrel dogs of both sexes were used. The methods of obtaining plasma and of intracutaneous injection have been described in Chapter III. In some of the experiments, histamine was also tested in the skin; 0.1 ml. of an isotonic solution containing 1 μg./ml. (calculated as the base) was injected. In the experiments testing the effects of the antihistamine mepyramine and of histamine depletion with compound 48/80, Pontamine Sky Blue (G. T. Gurr), 70 mg./kg. body weight was used for staining the plasma.* In the other experiments where blueing was necessary, T-1824 was used as described in Chapter III.

Use of an antihistamine. Mepyramine maleate (Neoantergan, Poulenc) was given intravenously as a 5% solution in isotonic saline.

Blued recipients were skin tested, and as soon as the initial injection sites had been read (i.e. approximately 30 minutes later), each recipient was given mepyramine, 2 mg./kg. body weight. Twenty minutes later the skin tests were repeated.

Use of a histamine liberator. Twenty-four to 48 hours before blueing, random sites were injected intracutaneously with 0.5 ml. of a 0.1% solution in isotonic saline of the histamine liberator compound 48/80

*These experiments were performed before most of the experiments in Chapter III, at a time when we were using Pontamine Sky Blue following the recommendation of Miles and Miles (1952). It was perfectly satisfactory for our purposes, but when experiments on valuable pure-bred dogs were proposed, it was felt safer to change to T-1824, whose toxicity has been more carefully studied, and to reduce the dose of dye to 5 mg./kg. Once this procedure was adopted, use of Pontamine Blue was discontinued in order to standardize all future experiments. We have had no reason to suspect that the cutaneous responses to plasma are any different when Pontamine Blue is used instead of T-1824 (compare, for example, Table VI with Table I).

(Burroughs Wellcome). The bleb raised by the volume of injected solution was demarcated with ink so that the treated area could be used again as an injection site after the dog had been blued.

Gastric acid secretion. Eight dogs, fasted for 24 hours, were transfused with 20 ml. of plasma per kg. body weight at a rate of 2 ml./kg./min., under sodium pentobarbital anaesthesia (approximately 25 mg./kg., given intravenously). The procedure for obtaining and handling plasma is described in Chapter V. Four animals were given autologous and four non-autologous plasma. Both series of animals were subjected to the same bleeding procedure and both types of plasma were obtained and treated in the same way.

The donors of non-autologous plasma, however, were deliberately selected on the basis of the blued recipients' cutaneous responses to intracutaneously injected dilutions of donor plasma. Where donor plasma failed to produce a wheal at a dilution of 1/9, this donor was rejected and replaced.

After induction of anaesthesia, the pylorus was exposed and gently tied off through a mid-line incision and a tube was introduced into the stomach through the esophagus. After thorough gastric lavage, 50 ml. of distilled water were placed in the stomach; every 15 minutes thereafter the fluid was aspirated off and replaced with a further 50 ml. of distilled water. The removed fluid was titrated with 0.05 N NaOH to pH 7 using phenol red indicator. In 8 titrations, the neutralized solutions as judged with the indicator were checked with a Beckman Model G pH meter. The measured pH values averaged 7.2 ± 0.1 (mean $\pm$ S.D.).

Free histamine. These experiments followed the transfusion experiments

described in Chapter V. The infrequency of hypotensive episodes following transfusion of non-autologous plasma suggested that if histamine is released at all, it is released in relatively small amounts. With this in mind, we developed a simple method for detecting the release of small quantities of histamine. Briefly, the method involves the intracutaneous injection of the suspected histamine liberator, followed by excision of the injected area and incubation in Tyrode's solution. Because of its high diffusibility and low affinity for tissue some or all of any histamine released should distribute itself in the incubation medium (Rocha e Silva and Schild, 1949; Mongar and Schild, 1952). This medium is then assayed for free histamine.

At the start of each experiment, a pair of dogs was selected at random. The recipient was blued, and samples of diluted plasma were tested on the skin. Where donor plasma failed to produce a distinct wheal at a dilution of 1/3, this donor was rejected and replaced.

After skin testing, the recipient dog was anaesthetized with sodium pentobarbital, 25 mg./kg., and a tracheotomy performed. 1.0 ml. of non-autologous plasma was injected intracutaneously at a site chosen at random along the mid-axillary line. When the first tinge of blue appeared at the site, the entire bleb raised by the injected plasma was rapidly undercut and excised with a sharp scalpel. The excised tissue consisted of both full-thickness skin and 2-3 mm. of subcutaneous tissue. This tissue was placed on filter paper, weighed, cut into slices approximately 2 mm. thick, and dropped with the filter paper into a test-tube containing 4.0 ml. of Tyrode's solution. The entire procedure was then repeated with autologous plasma, using an injection site immediately adjacent to that of the non-autologous injection. The time interval

between injection and excision was the same as that for non-autologous plasma. In each experiment, 3 pairs of injections were made, those of autologous plasma alternating with those of non-autologous plasma.

Each sample of excised, sliced tissue was allowed to incubate in Tyrode's solution at room temperature for 30 minutes. Then, following brief centrifugation, the supernate was pipetted off and treated with trichloroacetic acid to a final concentration of 10%. The resulting precipitate was removed by centrifugation, and 0.3 ml. of concentrated HCl and some boiling chips were added to the supernate. This solution was held in a bath of boiling saline (107°C.) for 90 minutes. The sample was then brought to pH 7.5±0.5 with NaOH and 0.2 ml. of 1 M Tris(hydroxymethyl)aminomethane buffer, pH 7.7. The pH was always checked with a Beckman Model G pH meter.

The neutralized samples were assayed for histamine on an isolated segment of guinea-pig ileum suspended in a 6 ml. bath filled with Tyrode's solution (of the composition recommended by Code and McIntire, 1956), containing 0.15 mg. of atropine sulphate per litre. A representative assay is shown in Figure VI. No more than 0.1 ml. of sample was added to the bath at a time, for larger additions sometimes had a depressant effect on the gut, probably owing to their hypertonicity. Because the amount of histamine in the samples often was small, intestine insensitive to 0.003 μg. was rejected and replaced. At the end of a series of assays, 0.020 μg. of mepyramine maleate was added to the bath. After several washings, some assays were repeated to test parallel inhibition of the histamine standard and samples. These tests never indicated the presence of a contracting substance other than histamine.

To ensure the validity of the method, the recovery of standard

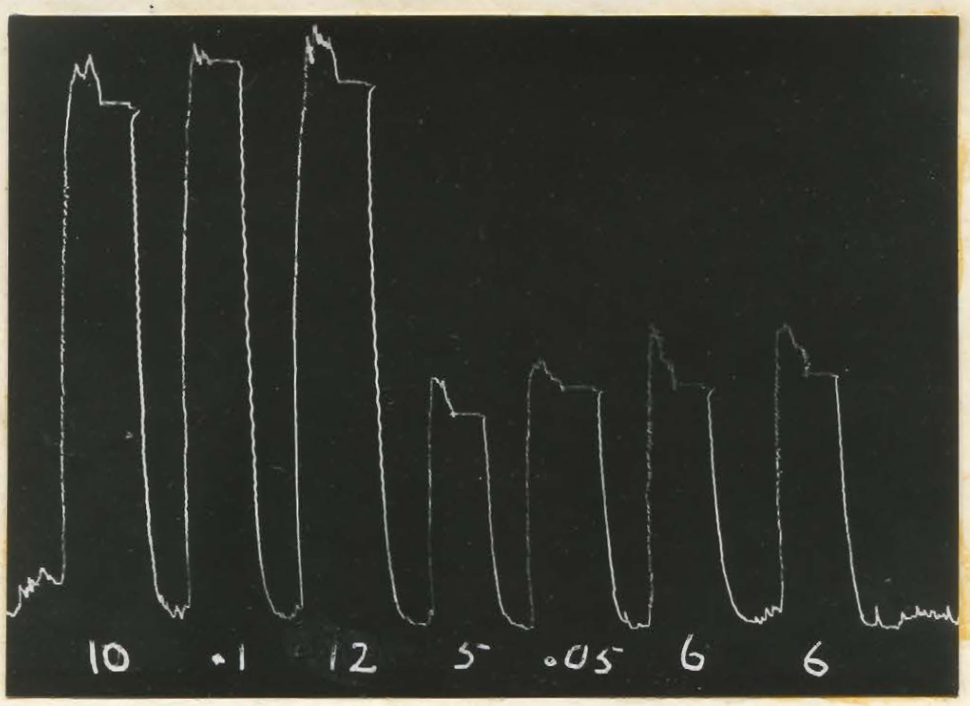


FIGURE VI - Histamine Assay on the Atropinized Guinea-pig Ileum.

The photograph shows the kymograph record of contractions of a segment of guinea-pig ileum. Actual size. Drum speed 9 mm./min. Contraction of the gut caused an upward swing of the stylus.

Preliminary screening of the samples had shown the particular sample assayed here to have a histamine content of about 0.1 µg./ml. From the left: the first, third, fourth, sixth, and seventh contractions were caused by 0.010, 0.012, 0.005, 0.006, and 0.006 µg. of histamine respectively, added to the bath as a solution containing 0.10 µg./ml.; the second and fifth contractions were caused by 0.10 and 0.05 ml. of sample respectively. The histamine content of the sample was recorded as 0.11 µg./ml.

TABLE V - Recovery of Histamine after Treatment with TCA, HCl and Heat.

Total Amount of Histamine		% Recovery
Initial	Final	
1.00 µg.	0.90 µg.	90
1.00	0.90	90
0.75	0.64	85
0.75	0.72	96
0.50	0.43	86
0.50	0.45	90
0.25	0.20	80
0.25	0.19	76
0.00	0.00	-
0.00	0.00	-

histamine solutions was tested. 1.00 ml. of an appropriate histamine solution in saline was added to 1.00 ml. of heparinized dog plasma. 4.00 ml. of Tyrode's solution were added. After 30 minutes at room temperature, the solution was treated with trichloroacetic acid, HCl, and heat, neutralized, and assayed, in exactly the same way as were supernates from the incubated tissue. The recoveries are shown in Table V; the mean recovery in 8 trials was 87±2(S.E.)%.

The amount of free histamine recovered per sample of skin, in µg., was calculated from the expression $\frac{A}{V} \times (4+S)$ where A = total histamine in assayed sample, in µg., V = volume of supernate removed after incubation, in ml., and S = weight of excised tissue, in g. (4 + S represents fairly closely the original volume through which any free histamine can distribute itself).

All values for histamine are expressed as the base.

Compound 48/80, dissolved in isotonic saline, was tested in the same way as plasma in some experiments. At the end of these experiments, the minimum dose of 48/80 which could produce a 10% fall in arterial blood pressure was determined. Blood pressure was recorded with a mercury manometer from the femoral artery; 48/80 was given intravenously in ascending doses at 5 minute intervals.

RESULTS

Effect of mepyramine. The results in 8 different recipients, each tested with a separate sample of non-autologous plasma, are shown in Table VI. A dose of 2 mg. of mepyramine per kg. body weight reduced the mean diameter of the blued area produced by non-autologous plasma from 12.6 mm. to 7.3 mm., and of that produced by histamine from 10.2 to 4.7 mm. The reduction in both cases was significant ($p < 0.01$ by

TABLE VI - Effect of Mepyramine Maleate on Cutaneous Responses to Non-Autologous Plasma and Histamine. Each value represents the mean from duplicate injections.

	Before Mepyramine				After Mepyramine			
	Autologous Plasma		Non-Autologous Plasma		Histamine 1μg./ml.		Non-Autologous Plasma		Histamine 1 μg./ml.	
	Diameter	Intensity	Diameter	Intensity	Diameter	Intensity	Diameter	Intensity	Diameter	Intensity
	n.m.*	-	11.8 mm.	2	10.8 mm.	3	2.0 mm.	-	2.0 mm.	-
	n.m.	-	16.2	4	5.3	2	10.8	3	2.0	-
	n.m.	-	12.5	4	11.8	4	2.0	-	3.6	1
	5.1 mm.	1	10.8	4	11.4	4	7.9	1½	2.0	-
	n.m.	-	12.0	4	10.0	4	9.8	2½	6.1	1
	n.m.	-	10.1	3	11.5	4	3.5	1½	6.5	1
	4.0	3	12.4	4	10.8	4	10.5	3½	6.4	2
	n.m.	-	15.3	4	10.0	4	12.2	3½	8.8	3
Mean			12.6		10.2		7.3		4.7	
± S.E.			±0.7		±0.7		±1.5		±0.9	

* n.m. : needle mark < 2 mm. in diameter.

t test using paired values).

Effect of compound 48/80. The effect of previous treatment with 48/80 is shown in Table VII. The response to non-autologous plasma was reduced on the average from 11.8 mm. in untreated areas to 8.2 mm. in treated areas ($p<0.01$, paired values). The response to 48/80 was reduced from 14.5 mm. to 10.0 mm. ($p<0.05$, unpaired values). The response of treated areas to histamine was not appreciably affected ($p>0.3$, paired values).

Gastric acid secretion. The results are shown in Figure VII. Autologous plasma transfusions were uniformly without effect on acid secretion. In contrast, every non-autologous plasma transfusion was followed by a rather sharp increase in secretion. In three dogs, the increase began within 30 minutes of the transfusion; in the fourth dog it was delayed for an hour. It is interesting that the recipient with the delayed response had a severe hypotensive episode following the transfusion, as described in Chapter V; the increase in acid secretion coincided with the return of the blood pressure to normal.

Free histamine. The results of 10 consecutive experiments are shown in Table VIII. The amount of free histamine recovered after non-autologous plasma injection was on the average 0.296 µg., after autologous plasma, 0.141 µg. Application of the *t* test to individually paired values showed this difference to be significant ($p<0.001$).

Two variables besides the source of the plasma should be considered: the amount of tissue excised (to which the amount of spontaneous histamine release is related), and the presence of small quantities of extravascular T-1824 in the tissue treated with non-autologous plasma. The weight of tissue excised after non-autologous injection was on the

TABLE VII - Effect of Previous Treatment with a Histamine Liberator on Skin Responses to Non-Autologous Plasma, Histamine, and Compound 48/80. Each value represents the mean from duplicate injections.

Recipient animal	Non-autologous Plasma				Histamine 1 µg./ml.				Compound 48/80 1 mg./ml			
	Untreated		Treated		Untreated		Treated		Untreated		Treated	
	Diameter of blued area	Intensity	Diameter of blued area	Intensity	Diameter of blued area	Intensity	Diameter of blued area	Intensity	Diameter of blued area	Intensity	Diameter of blued area	Intensity
No. 1	12.0 mm.	4	8.1 mm.	1½	10.0 mm.	4	10.5 mm.	3½	13.9 mm.	4	8.2 mm.	1
No. 2	10.1*	2½	8.8	½	11.5	4	11.2	3			9.2	2
	11.0*	3½	7.8	2								
No. 3	11.6	4	8.2	2½	10.8	4	10.4	3½	16.0	4	10.00	1
No. 4	14.1	4	8.2	2	13.2	4	10.8	3	13.5	4	12.5	1
Mean	11.8		8.2		11.4		10.7		14.5		10.0	
S.E.	±0.6		±0.2		±0.7		±0.2		±0.8		±0.9	

* Different donors.

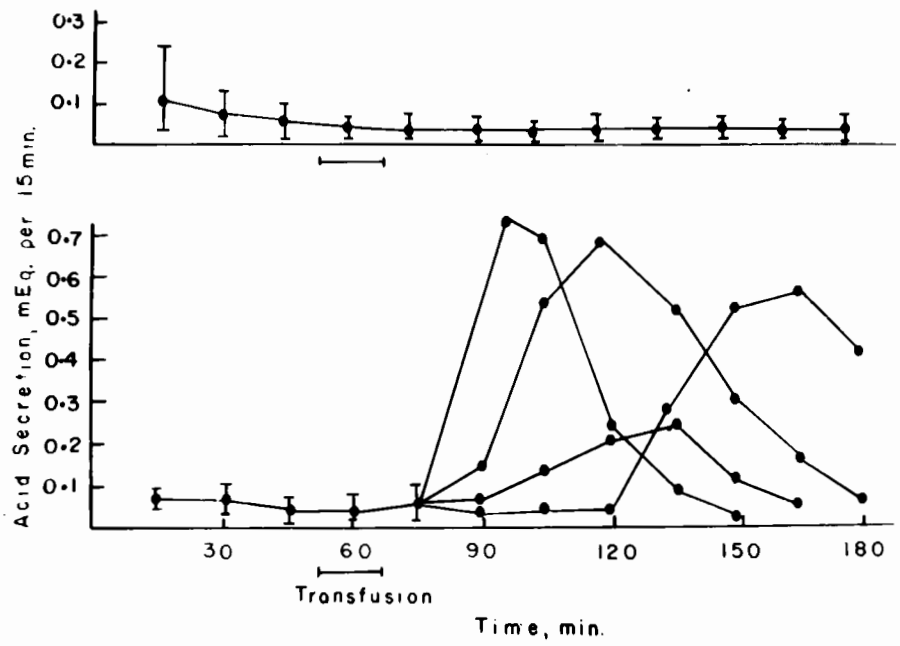


FIGURE VII - Effects of Plasma Transfusions on Gastric Acid Secretion.

The graphs show the total acid secretion of four dogs receiving autologous plasma (above) and of four dogs receiving non-autologous plasma (below). Closely grouped values are presented as a mean with full range.

TABLE VIII - Free Histamine Recovered after Plasma Injection.

	Non-Autologous Plasma		Autologous Plasma	
Expt. No.	Mean	S.E.	Mean	S.E.
1	0.49 ±	0.06 µg.	0.15 ±	0.03 µg.
2	0.55	0.06	0.30	0.01
3	0.33	0.04	0.14	0.03
4	0.26	0.05	0.14	0.05
5	0.25	0.01	0.06	0.01
6	0.15	0.03	0.06	0.02
7	0.12	0.01	0.12	0.02
8	0.15	0.01	0.15	0.01
9	0.21	0.01	0.11	0.01
10	0.45	0.05	0.18	0.02

TABLE IX - Free Histamine Recovered after 1.0 ml. Injections of Compound 48/80 and Autologous Plasma.

Conon. of 48/80, g./ml.	Expt. No. 6		Expt. No. 7	
	48/80	Plasma (adjacent area)	48/80	Plasma (adjacent area)
10^{-3}	0.23 µg.	0.09 µg.	0.43 µg.	0.10 µg.
10^{-4}	0.18	0.05	0.30	0.10
10^{-5}	0.16	0.04	0.23	)
10^{-6}	0.12	)	0.22	)0.15
10^{-7}	0.04	)0.04	0.13	)
Dose of 48/80 causing 10% fall in B.P.	0.2 mg. (0.025 mg./kg.)		0.5 mg. (0.025 mg./kg.)	

average 1.43 g., after autologous injection 1.38 g. This difference is not significant (S.E. of the difference = 0.05 g., $p > 0.3$), nor is it in any case large enough to be of importance. In experiments 9 and 10, T-1824 was not given, and tissue was excised after an interval arbitrarily set at 2½ minutes after injection; the difference in histamine release was as marked as in the other experiments.

Results obtained with compound 48/80, in the two experiments in which it was used, are shown in Table IX. The skin injection method was sensitive to 1/200 to 1/500 of the dose of 48/80 needed to cause an appreciable fall in blood pressure.

DISCUSSION

These results show that the effects of non-autologous plasma meet the requirements of the four criteria for histamine release set down in the introduction to this chapter. The validity of each of these criteria is discussed in Chapter II, but some comment on the particular conditions used in the present experiments is appropriate.

The reduction of the response to non-autologous plasma by the antihistamine mepyramine is by itself good evidence for a histamine-release mechanism. Mepyramine is noted for its high degree of specificity as an antihistamine. For example, Reuse found that its pA_2^* for the antagonism of the action of histamine on the guinea-pig ileum was 9.32, but only 5.03 for the antagonism of acetylcholine in the same system. That is, mepyramine, on a molar basis, was about 20,000 times

*The pA_2 is the negative logarithm to the base 10 of the molar concentration of the antagonistic drug which will reduce the effect of a double dose of the active drug to that of a single dose.

more effective against histamine than against acetylcholine. Furthermore, the dose of mepyramine used in the present experiments (2 mg./kg.) was not inordinately large, for, although it was sufficient to decrease the response to a moderate dose of exogenous histamine, it did not always abolish it.

The response to non-autologous plasma was much reduced in areas of skin previously injected with 48/80. Feldberg and Talesnik (1953) have shown that 48/80, in concentrations similar to that used here, reduced the histamine content of a treated area of dog skin by as much as 85% for at least 10 days. That the skin histamine was considerably reduced by 48/80 in the present experiments is indicated by the decreased effect of a second injection of 48/80 into the same site. That the capillaries in treated areas could still react by an increase of permeability is shown by the near normal response of these areas to exogenous histamine. Thus the reduction in the response to non-autologous plasma in these areas again suggests, although it does not prove, that at least part of the effects of non-autologous plasma can be attributed to a release of histamine.

The striking contrast between the effects of autologous and non-autologous plasma on gastric acid secretion is interesting from two points of view. First, it provides further circumstantial evidence that non-autologous plasma releases histamine. Second, it shows that if the increased secretion is indeed due to histamine, non-autologous plasma is able to release histamine on systemic as well as on intracutaneous injection.

The recovery of more free histamine from skin injected with non-autologous plasma than from skin injected with autologous plasma

makes the evidence conclusive that non-autologous plasma releases histamine.* The objection might be raised that in these latter experiments non-autologous plasma was not really releasing histamine but instead was having a leucotaxic effect, and that the increased amount of free histamine recovered from incubates of the excised skin was due to spontaneous release from increased numbers of histamine-rich leucocytes in the injected skin areas. Although we have not examined histologically sites injected with non-autologous plasma, this possibility is extremely remote. Leucocytic infiltration would have had only about two minutes to occur before excision of the skin. In addition, the leucocytes of the dog, in contrast to those of some other species, contain almost no histamine (Code, 1937, 1939). Another possible objection is that the present interpretation of the experiments involves the tacit assumption that the active substance being assayed on the guinea-pig ileum is in fact histamine. This assumption is almost certainly valid. The treatment of the skin extracts with trichloroacetic acid, hydrochloric acid and heat would inactivate such interfering substances as active proteins and 5-hydroxytryptamine. The assay itself was performed in the presence of atropine. The contraction produced by the extracts resembled in its time course that produced by standard histamine solutions, and the slope of the dose-response curve was identical for the extracts and for histamine (see Figure VI). Finally, mepyramine, in doses sufficient to reduce but not to abolish the gut's response to histamine, caused parallel

*As an incidental observation it might be mentioned that the method used for this demonstration seems to be well suited for the detection of histamine liberators of low potency, or of higher potency but available only in minute amounts. It detected histamine release by as little as 1 μg. of 48/80, a dose several hundred times less than the intravenous dose needed to cause hypotension. It has the further advantage of permitting the screening of many drugs for histamine-releasing properties in one animal.

inhibition of the effects of the sample and of standard histamine.

The recovery of some free histamine from skin injected with autologous plasma should not be interpreted as an actual release of histamine by such plasma. It is much more likely that this free histamine is a product of spontaneous release and the trauma of excision and slicing, for intracutaneous or intravenous injection of autologous plasma (Chapter V) rarely if ever causes any (untoward) reaction.

The present method needs further development before it can be used for a strictly quantitative measure of the total amount of histamine released. Some liberated histamine is probably removed by the circulation before excision of the injected tissue, and there is no absolute assurance that free histamine in the removed tissue reaches complete equilibrium with the incubation medium. Concerning the latter point, however, it is likely that equilibrium is closely approached, for Mongar and Schild (1952) found that with 100-200 mg. pieces of guinea-pig tissue incubated with various histamine liberators, 75% of the total histamine released into the medium occured in the first 10 minutes. In the present experiments, moreover, there is some reason for believing that non-autologous plasma releases only a small portion of the total skin histamine. The amount of release as estimated by our method is small ($<10\%$) relative to the total skin histamine content of dogs (Feldberg, 1956; Perry, 1956); in a few pilot experiments we were unable to find differences in the residual skin histamine after injection of autologous and non-autologous plasma; and in some further pilot experiments we found that 48/80 is still able to produce marked whealing in areas of skin previously injected with non-autologous plasma.

It should be remembered that the dogs used in the experiments on gastric acid secretion and on histamine release from injected skin did not represent a random sample, but a sample in which the degree of plasma incompatibility was somewhat more pronounced than in the general population of mongrel dogs. How representative of the general population these dogs were can be seen from Table II, which shows that in about 48% of random donor-recipient pairs the recipient shows a response to a dilution of 1/9 or higher of donor plasma (the criterion for the gastric secretion experiments), and in about 88% of random pairs the recipient shows a response to a dilution of 1/3 or higher (the criterion for the histamine release experiments). Whether higher percentages of random pairs would show increased gastric secretion or histamine release cannot be decided without further experiments. However, if the blueing after intracutaneous injection is another manifestation of the histamine-releasing property of non-autologous plasma, as it almost certainly is, then it is reasonable to conclude that if our method were sufficiently sensitive, almost every pair of dogs would show at least the histamine release from skin.

Although these experiments show that non-autologous plasma releases histamine, they do not exclude the possibility that part of the effect of non-autologous plasma is mediated through some other mechanism. Since the histamine release suggests (although it does not prove) that non-autologous plasma acts on the mast cells, a release of heparin and 5-hydroxytryptamine might be expected. If 5-hydroxytryptamine plays a role in the cutaneous reaction, however, it is probably a small one, for the 5-hydroxytryptamine content of dog skin is very low (0.1 ug./g., West, 1958). We are presently investigating whether

heparin is released after non-autologous transfusions. If it is, the evidence that non-autologous plasma acts on the mast cell will be considerably improved.

CHAPTER V

TRANSFUSION REACTIONS DUE TO PLASMA INCOMPATIBILITY IN DOGS

Untoward reactions are not infrequent following the transfusion of plasma or serum or whole blood in dogs. Freeman and Wallace (1938) noted that of 10 transfusions of dried reconstituted serum, three were followed by urticaria. Metcalf (1944) mentions occasional anaphylactoid reactions with hypotension and urticaria in dogs transfused with plasma or serum. Hamilton, Parkins and Waltzer (1947) found that urticaria often occurred after plasma infusions in dogs. Guyton, Lindley, Touchstone, Smith and Batson (1950), while studying the effects of massive transfusions of whole blood, were forced to discard the results in six out of 34 dogs because of 'transfusion reactions with resultant severe urticaria and fall in blood pressure'. Markowitz (1954) comments that blood transfusion reactions in dogs are characterized by shivering and vomiting and sometimes by anaphylactoid shock.

It will be recalled from the discussion in Chapter I of erythrocyte types in dogs that most of these reactions cannot be explained on the basis of erythrocyte incompatibilities. Their anaphylactoid nature suggested that the type of reactivity observed after intracutaneous injection of non-autologous plasma might be paralleled by a systemic reaction if plasma were given intravenously. The experiments described in this chapter were designed to test this possibility. Special attention was paid to blood volume changes, since usually the main reason for a transfusion, particularly a plasma transfusion, is to expand the blood volume.

Dr. D. G. Johns assisted with many of these experiments.

METHODS

Animals. Mongrel dogs of both sexes weighing 20-30 kg. were used for all experiments except those under anaesthesia where fewer blood samples were taken and smaller animals (5-10 kg.) could be used without undue interference with the blood volume. None of the animals had previously received any dog plasma or other injections.

The dogs were trained to lie quietly on an operating table so that only local anaesthesia was necessary during an experiment. The training was surprisingly successful: none of the subjects rebelled, and there was rarely any evidence that the experiments were being interfered with by alarm reactions.

Preparation and handling of plasma. Plasma was obtained by a two-stage bleeding on consecutive days from the lateral vein of the hind leg. One ml. of 2% xylocaine (without epinephrine) was injected subcutaneously in the region of the vein, and the vein was exposed. To ensure a free flow of blood, a polyethylene catheter large enough to accept a 16 gauge needle was introduced as far as the inferior vena cava. Twenty ml. of blood per kg. of body weight were removed on each occasion into heparin (100 mg./l.). On no occasion did signs of shock ensue. The blood was centrifuged at 2000 r.p.m. (r=20 cm.) for 10 minutes, the plasma aspirated off, and the cells, resuspended in isotonic saline, were reinfused into the animal. Chloramphenicol, 20 mg./l., was added to the plasma, which was stored at 4°C. It was never kept longer than 48 hours, and was always recentrifuged immediately before use.

Transfusions of plasma were made at a controlled rate through a

catheterized leg vein.

Determinations. All blood samples were obtained from, and all injections were made into, the indwelling venous catheter. Blood samples were taken into syringes which had been rinsed with a 1% heparin solution and dried in an oven. Before any sample was taken, 5 ml. of blood were withdrawn to rinse the catheter; this blood was returned to the animal after the sample had been obtained.

The circulating plasma volume was measured with T-1824 (Brickman), the concentration at zero time being determined by extrapolation back from the concentrations of four samples taken at $7\frac{1}{2}$ minute intervals after the injection of the dye. This timing was chosen for two reasons: 4 samples were considered necessary for accurate extrapolation, and Greve and Hamilton (1957) have shown that 30 minutes after injection the disappearance curve of T-1824 in dogs changes direction rather abruptly to become less steep. The plasma volume, in litres, was calculated by dividing the value in mg. of the amount of dye injected by the plasma concentration in mg./l. of dye at zero time. The dye was injected as a 0.20% solution in isotonic saline; 5 ml. were generally given per injection, except in a few very large dogs, where 6 ml. were given. The injection was made with a syringe held in a device for constant-volume delivery, and was followed immediately by a flushing injection of 5 ml. of isotonic saline introduced through a 3-way stopcock fitted between the dye syringe and the needle at the end of the catheter. The actual volume injected was determined by delivering an equivalent volume of water into a tared beaker and weighing. Dye concentrations were determined with a Beckman Model DU spectrophotometer at 620 mμ. after acetone extraction of the plasma (Chinard and Eder, 1948). The zero setting on

the spectrophotometer was made with a blank prepared by extraction of plasma taken just before the first dye injection. The extinction produced by each sample was compared with the extinction of extracted plasma samples made up to a known dye concentration with the same dye solution as that injected. The standards were always prepared from the plasma of the particular experimental animal, and care was always taken that the standards covered the same range of concentration as the post-injection samples.

Total plasma protein was determined in duplicate by the biuret method of Robinson and Hogden (1940). Samples containing T-1824 needed decolorization; this was achieved by the addition of Raney nickel-aluminum alloy as described by Stewart and Burgen (1958).

The hematocrit was determined in duplicate in Wintrobe tubes by centrifugation at 2500 r.p.m. for 30 minutes (r=10 cm.).

To show the accuracy of these methods: in 8 dogs, the mean individual difference, ignoring the sign, between two successive plasma volume determinations was 0.8 ± 0.2 (S.E.) ml/kg., between two successive plasma protein determinations was 0.16 ± 0.05 g.%, and between two successive hematocrits was 1.8 ± 0.7 percent (cells). The determinations were spaced 30 minutes apart.

In the few experiments in which it was measured, plasma hemoglobin was determined by the method of Flink and Watson (1942), which involves the conversion to pyridine ferrohemochromogen followed by colorimetric estimation. The method in our hands was able to detect 10 mg. of hemoglobin per 100 ml. of plasma.

Skin testing. An assessment of the sensitivity of the dog to the donor plasma was made by intradermal injection of 0.1 ml. of serial 3-fold

dilutions of the plasma in saline as described previously. These tests were performed after the initial plasma volumes had been determined with T-1824, so that the wheals were easy to distinguish by their blue tinge.

Erythrocyte cross-matching. Plasma and serum samples were examined for erythrocyte isoagglutinins by direct cross-matching. 0.05 ml. of a 5-10% suspension of erythrocytes in saline were added to 0.2 ml. of plasma or serum. After incubation at 37°C. for one hour, the suspension was examined microscopically for agglutination. Cross-matching was also performed in a high-albumin medium according to the directions of Hamilton (1948).

For convenience, the design of each experiment is described with the results.

RESULTS

Group 1. Comparison of the plasma-expanding efficiency of autologous and non-autologous plasma. Eight dogs were divided into four pairs of matching weight, were bled, and on the day following the second bleeding were transfused with 20 ml. of plasma/kg. at the rate of 1 ml./kg./minute. The animals were unanaesthetized. Two pairs received autologous plasma and two non-autologous plasma. Two weeks later the entire procedure was repeated with the types of transfusion - autologous and non-autologous - reversed, so that at the end of the experiment each animal had received both an autologous and a non-autologous transfusion. The procedure for each transfusion is outlined in Table X.

To our knowledge the source of the plasma was the only variable in these experiments. The recipients of both autologous and non-autologous transfusion had undergone identical bleeding schedules and were entirely

TABLE X - Procedure for each Transfusion of Group I.

Day 1. Plasma volume, plasma protein, and hematocrit determinations. Dog bled of 20 ml./kg. Cells replaced.

Day 2. Dog bled of 20 ml./kg. Cells replaced.

Day 3. Time, min.	0	Plasma volume, plasma protein, and hematocrit determinations.
	30	Plasma volume, plasma protein, and hematocrit determinations. Skin testing.
	60-80	Transfusion of 20 ml./kg. of autologous or non-autologous plasma.
	110	Plasma volume, plasma protein, and hematocrit determinations.
	140	Plasma volume, plasma protein, and hematocrit determinations.

comparable in initial blood volume and hematocrit. The samples of autologous and non-autologous plasma were obtained, stored, and administered in the same way.

In Table XI the values for plasma volume, red cell volume, and plasma protein are given for the animals before bleeding and before transfusion. The plasma volume was reduced on the average by 7.6% and the circulating plasma protein by 16.6% as a result of the bleeding. However, these reductions were small relative to the volume and plasma protein content of the subsequent transfusions.

Within 15 minutes after transfusion of non-autologous plasma every animal developed cutaneous whealing, particularly in the neck and groin regions. The wheals were bright blue in colour owing to the leakage of plasma stained with T-1824, and were preceded and surrounded by erythema. In the more severe reactions, the wheals were generalized and confluent and facial edema occurred, particularly in the muzzle and periorbital tissues. The severe reactions were accompanied in some animals by vomiting and lethargy. On the other hand, no cutaneous or other reactions were seen after any of the autologous plasma transfusions.

Table XII gives the values for changes in plasma volume, protein and red cell volume following transfusion. Table XIII shows pertinent individual results. Retention of transfused volume within the circulation was complete after autologous plasma, but after non-autologous plasma only 51.5±10.3% of the transfused volume remained in the circulation 30 minutes after the transfusion. No significant further loss occurred during the subsequent 30 minutes. The estimated red cell volumes remained so constant throughout the experiments that the change in hematocrit provided almost as accurate a measure of the degree of

TABLE XI - Effect of the Bleeding Procedure on the Plasma Volumes, Cell Volumes, and Total Plasma Proteins of Eight Normal Dogs*.

	Pre-bleed	Pre-transfusion
a. Plasma volume (ml./kg.)	48.5 ± 1.9+	44.8 ± 1.2 ($p < 0.02$)‡
b. Cell volume (ml./kg.)	34.5 ± 1.5	33.5 ± 1.7 ($p = 0.3$)‡
c. Plasma protein (gm./kg.)	3.25 ± 0.12	2.71 ± 0.09 ($p < 0.001$)‡

* Two procedures per dog, spaced two weeks apart.

+ Mean ± standard error.

‡ Value for t, pre-bleed vs. pre-transfusion, paired values.

TABLE XII - Effect of Autologous and Non-Autologous Plasma Transfusions on the Plasma Volumes, Red Cell Volumes, and Total Plasma Protein of Eight Dogs.

		Autologous	Non-Autologous
a. Pre-transfusion plasma volume (ml./kg.)	60 min. before	45.0 ± 1.5*	45.0 ± 2.0
	30 min. before	44.2 ± 1.8	44.6 ± 2.1
b. Post-transfusion plasma volume (ml./kg.)	30 min. after	65.7 ± 2.1	55.3 ± 3.0
	60 min. after	65.2 ± 2.6	54.9 ± 3.1
c. Plasma volume expansion+ (as percentage of volume transfused)	45 min. after	104.0 ± 8.9	51.5 ± 10.3 ($p < 0.002$)‡
d. Plasma protein expansion+ (as percentage of amount transfused)	45 min. after	126.1 ± 10.8	58.9 ± 10.1 ($p < 0.001$)‡
e. Red cell volume+ (as percentage of pre-transfusion red cell volume)	45 min. after	99.8 ± 4.3	99.5 ± 6.6

* Mean ± standard error.

\+ Averaged for each animal from 30 and 60 minute pre- and post-transfusion determinations.

‡ Value for t, autologous vs. non-autologous, paired values.

TABLE XIII - Efficiency of Autologous and of Non-Autologous Plasma as Plasma Volume and Protein Expanders.

Dog No.	Plasma Volume Expansion* (as % of volume transfused)		Plasma Protein Expansion* (as % of amount transfused)	
	Autologous Transfusion	Non-Autologous Transfusion	Autologous Transfusion	Non-Autologous Transfusion
1	159%+	71%	191%+	79%
2	93+	13	119+	22
3	96+	33	105+	34
4	77+	16	97+	19
5	102	93+	126	77+
6	83	66+	99	88+
7	115	70+	138	76+
8	102	54+	134	76+

* Averaged from 30 and 60 min. pre- and post-transfusion determinations.

+ Denotes first of the two transfusions each animal received.

plasma expansion as did the dye dilution method.

After the non-autologous transfusions, much of the equivalent of the transfused plasma protein was lost from the circulation, but with autologous transfusions the increase in circulating protein appeared to be slightly greater than that transfused.

Plasma volume, total plasma protein, and red cell volume did not change with time in three control experiments in which the protocol was identical with the above experiments, but in which transfusion was omitted. The mean value after what ordinarily would have been the transfusion period, expressed as a percent of the mean value before the period, was 100.5±1.7 (S.E.)% for plasma volume, 100.3±1.6% for plasma protein, and 96.1±5.0% for red cell volume.

Group 2. Effect of mepyramine on the non-autologous plasma reactions. Plasma was obtained from eight pairs of dogs of matched weights by the standard bleeding procedure. Each member of a pair served as donor for the other, so that all animals received only non-autologous transfusions. Twenty ml. of plasma per kg. were transfused at the rate of 1 ml./kg./minute. No anaesthesia was used. One member of each pair received mepyramine, 5 mg./kg., as a 1.5% solution intravenously in divided doses 30 and 15 minutes before transfusion. The other member served as a control. The timing of the pre-transfusion determinations was different from that in the experiments in Group 1; determinations were made at 80 minutes and 50 minutes before transfusion. This allowed time for the mepyramine to act without interfering with the skin testing after the second dye injection.

None of the animals treated with mepyramine developed wheals following transfusion, whereas seven of the eight untreated animals

developed wheals similar to those already described. Table XIV shows that expansion of plasma volume and retention of plasma protein were considerably greater in the treated animals than in the untreated, but still were significantly less than the complete expansion found after autologous transfusion ($p < 0.02$ for both volume and protein expansion).

In three supplementary control experiments, mepyramine alone (i.e. in the absence of transfusion) did not affect the plasma volume or circulating protein. The mean value after what ordinarily would have been the transfusion period, expressed as a percent of the mean value before the period, was 100.3 ± 1.4 (S.E.)% for plasma volume, and 100.5 ± 0.9% for plasma protein.

Group 3. Effects of transfusions on blood pressure. Eight dogs were transfused under anaesthesia (intravenous sodium pentobarbital, approximately 25 mg./kg.) with 20 ml. of plasma per kg. at a rate of 2 ml./kg./minute. Arterial blood pressure was recorded from a femoral artery with a mercury manometer. Four animals were given autologous and four non-autologous plasma. Again, both series of animals were subjected to the same bleeding procedure and both types of plasma were obtained and treated in the same way. The donors of non-autologous plasma, however, were deliberately selected on the basis of the recipients' responses to intracutaneously injected dilutions of donor plasma: only those donors whose plasma produced a wheal at a dilution of 1/9 or higher were used. (The effect of transfusion on gastric acid secretion, already described in Chapter IV, was determined in these experiments).

The four animals receiving autologous plasma showed slight pressor responses. Three of the four dogs receiving non-autologous transfusions showed similar responses, but the fourth animal developed a severe hypotension, the mean blood pressure falling from 115 mm. Hg. to

TABLE XIV - Effect of Non-Autologous Plasma Transfusions on the Plasma Volumes, Red Cell Volumes, and Total Plasma Protein of Eight Untreated Dogs and of Eight Dogs Treated with Mepyramine Maleate.

		Treated	Untreated
a. Pre-transfusion plasma volume (ml./kg.)	80 min. before	50.4 ± 3.1*	48.0 ± 2.5
	50 min. before	50.6 ± 2.9	47.6 ± 2.6
b. Post-transfusion plasma volume (ml./kg.)	30 min. after	65.9 ± 3.5	56.8 ± 3.3
	60 min. after	63.7 ± 3.6	55.0 ± 3.4
c. Plasma volume expansion+ (as percentage of volume transfused)	45 min. after	71.5 ± 7.8	40.5 ± 8.9 ($p<0.03$)‡
d. Plasma protein expansion+ (as percentage of amount transfused)	45 min. after	83.8 ± 9.4	45.2 ± 10.6 ($p<0.02$)‡
e. Red cell volume+ (as percentage of pre-transfusion red cell volume)	45 min. after	105.0 ± 2.6	108.1 ± 8.7

* Mean ± standard error.

\+ Averaged for each animal from 80 and 50 minute pre-transfusion and 30 and 60 minute post-transfusion determinations.

‡ Value for t, treated vs. untreated, unpaired values.

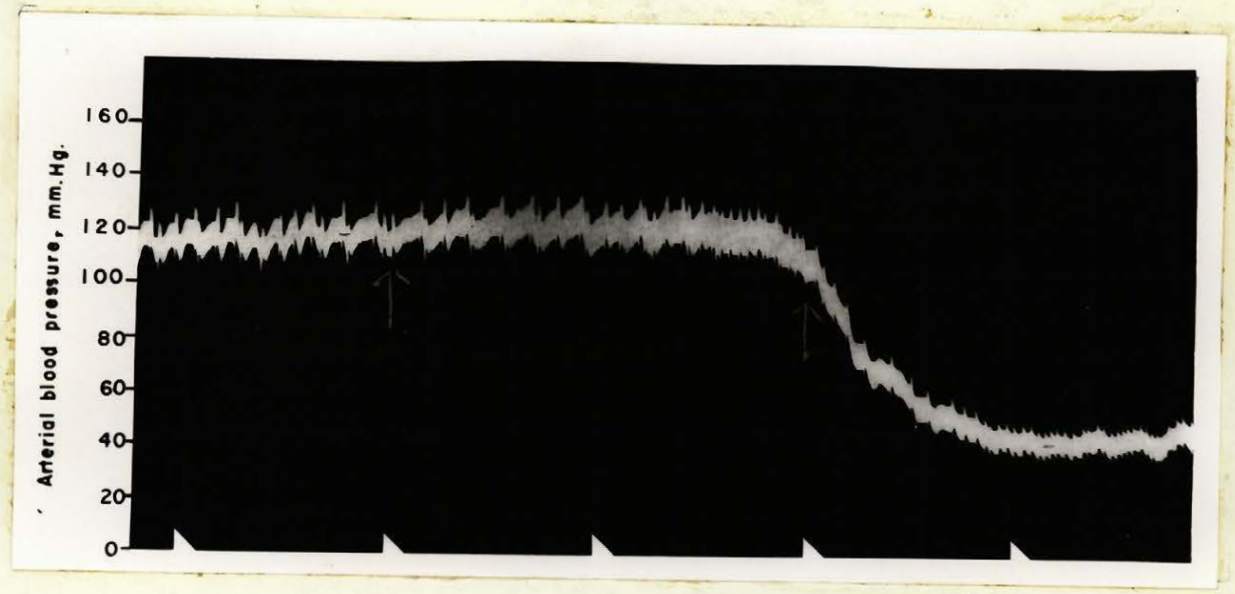


FIGURE VIII - Hypotension Following a Non-Autologous Plasma Transfusion.

The record is from a 7.7 kg. dog. Five minute intervals are marked on the base-line. Between the arrows 154 ml. of non-autologous plasma were transfused at a constant rate.

TABLE XV - Intradermal Titre, Whealing Reaction, and Plasma Expansion in Dogs Receiving Non-Autologous Plasma Transfusions.

Pre-Transfusion Intradermal Titre*	Whealing after Transfusion+	Plasma Volume Expansion, 45 minutes after transfusion, as percentage of volume transfused
1/27	4.0‡	19%‡
	(4, 4, 4)	(33, 16, 9)
1/9	2.7	51%
	(3, 3, 2)	(56, 19, 78)
1/3	2.1	58%
	(3, 1, 3, 3, 2, 1/2)	(26, 70, 60, 71, 54, 65)
Undiluted	1.4	47%
	(4, 1/2, 1, 0)	(13, 66, 93, 16)

* Highest dilution producing a detectable response.

\+ Graded on an arbitrary scale: 0 - no whealing; 1 - one or two wheals; 2 and 3 - moderate whealing; 4 - confluent whealing with severe facial edema.

‡ Mean value. Individual values in brackets.

40 mm. Hg. near the end of the transfusion and then remaining low for almost an hour. Part of the kymograph record is shown in Figure VIII. Similar hypotensive responses to non-autologous plasma have been observed in several pilot experiments.

Skin testing. It is of some interest to know whether the intensity of the intradermal reaction to plasma reflects the degree of whealing and of deficient retention after plasma transfusion. In Table XV are summarized the results in 16 dogs: the 8 dogs of Group 1 and the 8 control animals receiving non-autologous transfusions in Group 2. There is a fairly good correlation between the severity of whealing and the titre of the intradermal reaction. The correlation of either of these with retention of transfused plasma is poor.

Erythrocyte isoagglutinins. Immediately before ten of the non-autologous transfusions the sera of both donor and recipient were tested for erythrocyte isoagglutinins. None were detected. Further evidence that the reactions to non-autologous plasma are unrelated to any erythrocyte factors was obtained from two pairs of dogs which had shown strong reactions to each other's plasma. Four weeks after plasma transfusion, these dogs were transfused with 100 ml. of each other's washed erythrocytes, suspended in 100 ml. of isotonic saline. No signs of whealing, collapse or other untoward reaction occurred. Plasma hemoglobin determinations taken before and at hourly intervals for 6 hours after transfusion gave no evidence of a hemolytic reaction; the post-transfusion levels on no occasion were higher than the pre-transfusion levels.

DISCUSSION

These results show that in the unanaesthetized dog relatively large transfusions of non-autologous plasma almost always produce minor to moderate reactions and occasionally produce major reactions of an

anaphylactoid type. Although urticaria is the commonest manifestation of these reactions, a more significant feature is a loss of the equivalent of much of the added plasma within 30 minutes of transfusion. This loss is on the average sufficient to reduce the increment in plasma volume to about half the predicted value; occasionally it is so pronounced that the plasma volume is not expanded at all.

Autologous plasma is far superior to non-autologous plasma as a plasma volume expander. This conclusion is valid whether one compares the results of autologous transfusion with those of non-autologous transfusion in Group 1, or with those of non-autologous transfusion in the untreated animals of Group 2. The efficiency of autologous plasma as a volume expander, and the absence of reactions following its use, provide valuable evidence that the reactions to non-autologous plasma described here are not due to the methods of handling or storing the plasma but to a genuine incompatibility. Remington and Baker (1959) have recently confirmed these results; they found a high incidence of reactions to homologous plasma, characterized by urticaria, falls in arterial blood pressure, and poor expansion of the blood volume; autologous plasma transfusions produced the predicted increase in plasma volume, and were not followed by untoward reactions.

The plasma incompatibility demonstrated by transfusion is almost certainly another manifestation of the individual specificity of dog plasma. We can find no evidence to connect the incompatibility with the presence of natural erythrocyte isoagglutinins in the plasma: the occurrence of these isoagglutinins has been reported to be infrequent, as discussed in Chapter I, and in our own experiments we have been unable to demonstrate either natural isoagglutinins or pathological reactions to red cell infusions. The reactions to non-autologous plasma

transfusions again appear to be the result of a release of endogenous histamine. It was shown in Chapter IV that a sharp increase in gastric acid secretion often follows non-autologous transfusion. In the present experiments the urticarial reactions were completely inhibited and the expansion of the plasma volume was significantly improved by pre-treatment with an antihistamine. Similarly, Baker and Remington (1958) have found that the facial edema and fatal circulatory collapse which occur after non-autologous transfusions in adrenalectomized dogs can be prevented by the previous administration of the antihistamine diphenhydramine (Benadryl). The failure of mepyramine in our experiments to prevent completely the plasma extravasation while preventing the urticaria may mean that part of the reaction to non-autologous plasma transfusion is mediated by some mechanism other than histamine release. It is just as reasonable, however, to attribute this failure to the known resistance to the antihistamines of the cardiovascular effects of the histamine liberators (Paton, 1957).

It is obvious from the whealing reaction that some of the plasma loss from the circulation following non-autologous transfusion occurs into the skin. However, this is probably not the only site of plasma extravasation. Skin lesions and blueing no longer appear after treatment with mepyramine, yet some plasma loss still occurs. Although the present experiments do not show where this extra loss happens, the results of Deavers, Huggins and Smith (1957) suggest that it is not restricted to a particular tissue or organ. They found a generalized increase in the amount of 'trapped' (i.e. stagnant or only slowly circulating) plasma in the abdominal organs, lungs, and skeletal muscle, as well as in the skin, following massive transfusions of homologous blood in the dog.

Donor plasma was tested in the skin before transfusion to determine whether one could predict the outcome of transfusion of that plasma. The results of skin-testing were only of limited value in this respect. Although dogs which showed marked skin sensitivity to donor plasma also had severe reactions to subsequent transfusion, dogs with weak skin responses had reactions of variable, unpredictable severity. This lack of good correlation is not surprising. In addition to the difficulty of objective assessment of both skin responses and transfusion reactions, it is probable that the skin sensitivity to donor plasma is only one of several factors which determine the severity of the reaction to transfused plasma. Another factor, for example, may be the initial rate at which non-autologous protein reaches the extravascular space and the tissue mast cells.

Although few of our transfusions were performed under anaesthesia, we gained the impression that whealing was less prominent than in conscious animals. Similarly, Baker and Remington (1958) have reported that facial edema is less severe under anaesthesia after transfusion in adrenalectomized dogs, and in their most recent experiments (Remington and Baker, 1959) have found that the frequency of hypotensive episodes after transfusion is reduced by anaesthesia. The fact that many investigators have not reported serious transfusion reactions in dogs may be due to this effect of anaesthesia and to the wheals being less obvious when dye is not present in the plasma.

The physiological significance of these experiments is discussed in Chapter VII.

CHAPTER VI

EXPERIMENTS WITH OTHER SPECIES

Although only a few species have been examined for a plasma specificity similar to that of the dog, the results are interesting enough to warrant some description.

METHODS

Homologous plasma was tested in the skin of cats, rabbits, guinea-pigs, and man.

Light-coloured cats, rabbits and guinea-pigs of both sexes were closely clipped to remove the fur; in guinea-pigs, clipping was followed by the use of a depilator (Miles and Miles, 1952). Blood was obtained by cardiac puncture under light ether anaesthesia, and the animals were blued by intraperitoneal injection of Pontamine Sky Blue (G. T. Gurr), 70 mg./kg. Skin-testing was performed an hour later, after complete recovery from the anaesthetic. The techniques of handling and injecting the plasma were as described for the dog.

The human subjects were healthy young adult males. All apparatus was sterilized by dry heat. Blood was obtained by venipuncture. The subjects were blued by intravenous injection of 50 mg. of T-1824 (Warner-Chilcott), a dose which produced moderate staining of the plasma but left the skin colour normal during the experiment so long as capillary permeability was not increased. (A day or two after the dye injection, the skin of some subjects developed a faint grayish hue which persisted for about a week). Plasma was prepared and tested in the skin of the back in the same way as it was in the dog. Histamine, 1 μg./ml. (calculated as the

base) in isotonic saline, was also injected in 0.1 ml. volumes in some subjects for purposes of comparison. Cutaneous responses were always assessed by an independent observer unaware of the kinds of fluid injected at particular sites. Erythrocyte types were determined by slide tests using standard typing sera (Hartz).

RESULTS

Only in man were the results of more than routine interest. In rabbits (six donor-recipient pairs) no reaction was obtained to autologous or non-autologous plasma. In cats, no selective response could be demonstrated in six donor-recipient pairs, as the majority of recipients showed pronounced reactions both to autologous and to non-autologous plasma. In guinea-pigs there was a slight indication of a selective response, for two out of eight donor-recipient pairs showed moderate reactions to non-autologous plasma, while no recipients reacted to autologous plasma.

In human subjects, intracutaneous non-autologous plasma produced on the average larger reactions than autologous plasma, although the results were less uniform than in the dog. Typical responses are shown in Figures IX and X. In 16 donor-recipient pairs (eight donors tested in five recipients) the diameter of the blued area produced by non-autologous plasma averaged 8.0 mm., with a mean intensity of 2½. These lesions were all typical triple responses with well-marked flares, and they developed within five minutes of injection. Autologous plasma generally produced no more blueing than could be accounted for by the needle mark. It may be seen from Table XVI that of the 16 donor-recipient pairs, 11 showed clearly positive reactions to non-autologous

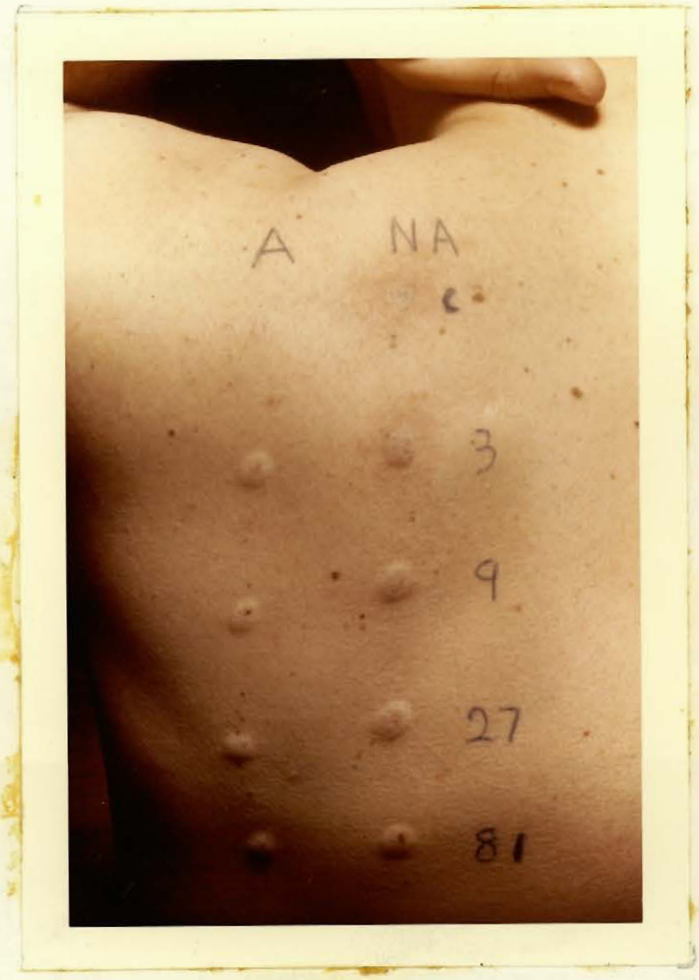


FIGURE IX - Responses of Human Skin to Autologous and Non-Autologous Plasma.

The left member of each horizontal pair of blebs represents the injection site of autologous plasma. At the uppermost sites, directly beneath the letters 'A' and 'NA', the plasmas were injected undiluted. At the other sites, the plasmas were first diluted with isotonic saline; the reciprocals of the dilutions are indicated.

The photograph was taken about one hour after T-1824 injection, and 30 minutes after the intracutaneous injections.

(Photograph by Dr. S. Freedman.)

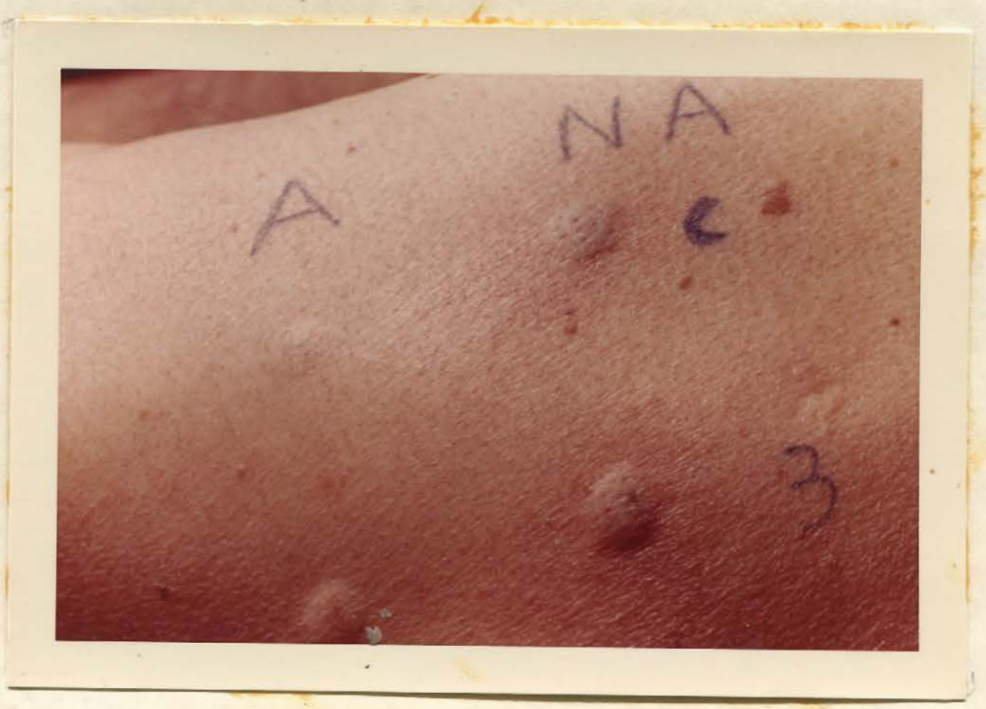


FIGURE X - Responses of Human Skin to Autologous and Non-Autologous Plasma.

This photograph is a close-up of the upper two pairs of responses shown in Figure IX. The reddish discoloration in the lower right was caused by shadow.

(Photograph by Dr. S. Freedman.)

TABLE XVI - Responses of Human Skin to Autologous and Non-Autologous Plasma and Histamine.*

	Blood type ABO	Blood type Rh	Autologous plasma: Diameter of blued area	Non-autologous plasma: Diameter of blued area	Non-autologous plasma: Intensity	Histamine 1 µg./ml.: Diameter of blued area	Histamine 1 µg./ml.: Intensity
Recipient No. 1	B	d	n.m.+			9.0 mm.	3
Donor No. 1	A	D		7.8 mm.	2		
Donor No. 2	0	D		12.3	4		
Donor No. 3	0	D		3.3	2		
Donor No. 4	A	D		10.8	3		
Recipient No. 2	A	D	n.m.				
Donor No. 2	0	D		15.2	4		
Donor No. 5	0	D		13.0	3		
Donor No. 6	A	D		13.5	3		
Donor No. 7	0	D		13.3	4		
Recipient No. 3	A	D	n.m.			11.5	3½
Donor No. 2	0	D		7.8	2½		
Donor No. 3	0	D		1.0			
Donor No. 6	A	D		3.6			
Donor No. 8	A	D		4.9	1		
Recipient No. 4	A	D	4.7 mm.			11.0	4
Donor No. 1	A	D		4.0	1		
Donor No. 6	A	D		1.0			
Recipient No. 5	0	D	n.m.			10.5	4
Donor No. 2	0	D		6.8	1		
Donor No. 7	0	D		10.6	2		

* Each value represents the mean from duplicate injections of 0.1 ml.
+ n.m. : needle mark < 2.0 mm. in diameter.

plasma and five gave doubtful reactions or none at all. The one recipient (No. 4) who reacted as strongly to his own as to donors' plasma had mild dermatographia, and his positive responses may have been due to trauma alone.

No correlation was found between ABO and Rh blood group incompatibility and reactivity to non-autologous plasma.

One subject (No. 5) was injected intracutaneously at two sites with 0.5 ml. of a 0.02% solution of compound 48/80 in isotonic saline 42 hours before blueing and testing. The responses to two different samples of non-autologous plasma were reduced from diameters of 10.6 mm. and 6.8 mm. in untreated areas to nothing more than needle marks in treated areas.

The testing in man of serial saline dilutions of plasma produced curious results. Dilutions of the order of 1/9 or higher often caused rapid blueing, but no flare, at the injection site. Since this effect occurred both with diluted autologous and with diluted non-autologous plasma, no further attempt was made in man to determine the titre of non-autologous activity by the testing of serial dilutions. It was found, however, that addition of soya bean trypsin inhibitor (Worthington), 0.1 mg./ml. of diluent, effectively prevented the blueing response to diluted autologous plasma. This response was also reduced when particular care was taken to inject samples immediately after their dilution (as was done in the experiment represented by Figures IX and X).

DISCUSSION

These results suggest that individual specificity of plasma may not be a phenomenon restricted to the dog. Although the few experi-

ments with cats, rabbits and guinea-pigs were unsuccessful in demonstrating any appreciable difference between the effects of autologous and non-autologous plasma, the experiments with human subjects showed that the cutaneous response to non-autologous plasma resembles that of dog skin, but is obtained with less regularity. Earlier workers (Chant and Gay, 1927; Levine and State, 1942; Maumsell, 1944), though they did not have the advantage of the blueing technique, have also reported cutaneous reactions to non-autologous plasma in man, as discussed in Chapter I.

Using the techniques described here, Dr. S. Freedman has continued the experiments on human subjects at the Montreal General Hospital. His results are in complete agreement with those reported here: in 38 recipients, the cutaneous responses to non-autologous plasma were clearly more pronounced than those to autologous plasma; in the remaining 9 recipients tested, both kinds of plasma produced either equal responses or no responses; in no recipient was the response to autologous plasma greater than that to non-autologous plasma. Every donor-recipient pair had the same major blood types.

It is difficult to decide how closely the phenomenon in man parallels that in the dog. The degree of specificity does not appear to match that of dog plasma, but the greater variability of the responses in man may be due in part to the fact that dogs were given much more dye before testing than were human subjects. In addition, it may be that the skin is not the ideal site in man for demonstrating the specificity of plasma. Whether human non-autologous plasma produces its effects by releasing histamine also remains undecided, although the effect of

pretreatment with 48/80 in the one experiment in which it was tried suggests that this may be the case. Even more suggestive is the simple observation that the response to non-autologous plasma is a triple response, for in man, only histamine or histamine-releasing substances are known to produce triple responses on intracutaneous injection (Paton, 1957).

The observation that diluted autologous plasma is able to produce blueing on intracutaneous injection in man was relevant to the present study only to the extent that it showed the danger in using serial dilutions for assessing non-autologous activity without careful controls. The observation did have considerable significance in another respect, however, for it suggested that simple dilution activates a permeability factor in human plasma in the same way as it does in guinea-pig serum (Miles and Wilhelm, 1955). This idea was confirmed in a series of experiments reported elsewhere (Stewart and Bliss, 1957).

The significance of the demonstration of human plasma specificity is discussed in Chapter VII.

CHAPTER VII

A GENERAL DISCUSSION

In the preceding four chapters, the validity of the experimental results and of the conclusions drawn from them has been discussed. In this chapter the wider significance of the results is considered.

A. The General Significance

The two most striking features of the individual specificity of dog plasma are first, the immediacy with which it manifests itself on injection, and second, its unusual degree of specificity. In adult animals, only rarely was it difficult to distinguish between non-autologous and autologous plasma within five minutes of their injection into the skin, and within 30 minutes one could always make the distinction. This was true whether the animals were mongrel or closely inbred pure-bred dogs. Concerning the degree of specificity, all that can be said is that the plasmas of the most closely related animals available for study were as easily identified with their donors as were plasmas from mongrel dogs. The most closely related animals we tested (Basenjis U and V, and Springers O-U, Figure III) were sufficiently inbred to be heterozygous for an average of only 10 per cent* of the loci at which heterozygosity existed in the original pure-bred population. The original population, moreover, since it was pure-bred, was considerably more genetically homogenous than the population of mongrel dogs. This is particularly true of the Basenjis, which represent an ancient breed

*Calculation by Dr. J. L. Fuller, Bar Harbor, Maine.

of dog. We can conclude, then, that the degree of specificity is extreme, although we cannot say how extreme until we find a 'compatible' pair of dogs either by chance or by continued inbreeding.

Although these two aspects, the immediacy and the specificity, make the phenomenon interesting, they make its interpretation difficult. On the one hand, the immediate release of histamine makes it tempting to postulate an immunological mechanism, but to do so requires some explanation of the absence of a latent period during which antibodies can form. On the other hand, the individual specificity suggests a genetic basis , but any genetic interpretation involves the assumption that sufficient inbreeding would attenuate the phenomenon, an assumption for which there is no experimental support. In other words, any interpretation must for the present be highly speculative. It may be worthwhile to so speculate, however, if only to show that whatever mechanism underlies the specificity of plasma must be an interesting one.

It is appropriate to refer first to the immediate effects of heterologous serum, which resemble, superficially at least, some of the effects of non-autologous plasma in the dog (Chapter I), and to the histamine-releasing ability in some species of such foreign proteins as egg white.(Chapter II). A glib interpretation of the individual specificity of dog plasma is to make it analogous to one or both of the above phenomena, with the dissimilarity between autologous and non-autologous dog plasma being merely lesser in degree, but resting on a similar chemical basis. The possibility that the phenomena are related cannot be rejected, although it is difficult to reconcile it with the absence of attenuation of the response to non-autologous plasma in closely

related dogs. Another difficulty is that Feldberg and Schachter (1952), and Schachter and Talesnik (1952), were unable to demonstrate histamine release by horse serum or egg white in non-sensitized dogs; it is entirely possible, however, that their method was not sensitive enough to detect the release of small amounts of histamine; moreover, since perfused skin flaps were used in their experiments, it may be that in the dog insufficient amounts of the foreign protein reached the tissue cells. In our own experiments we detected only rather small amounts of histamine released by non-autologous plasma, even though the plasma was injected directly into the skin to ensure free access to the tissue cells. The most serious difficulty, however, is in gaining much useful information by comparing the effects of non-autologous plasma in the dog with the initial effects of heterologous sera and protein, for no one has provided an explanation based on experimental evidence for the latter phenomena.

In the discussion which follows, possible mechanisms to account for the individual specificity of dog plasma are postulated; any one of these mechanisms might apply just as well to the actions of heterologous sera and protein.

Immunological interpretations. One immunological interpretation of the individual specificity of dog plasma is to attribute it to the presence of soluble antigen-antibody complexes in the plasma. It was pointed out in Chapter II that it has recently been found that under suitable conditions such complexes can produce the manifestations of an antigen-antibody union within the body, with histamine release. It is conceivable that each dog has unique immunological experiences during his lifetime,

and that these experiences provide him with circulating antigen-antibody complexes specific to himself. Presumably an animal would in some way become tolerant to his own complexes. When exposed to another animal's complexes, however, an immediate reaction, with histamine release, might be expected. This idea will be easier to evaluate when more is known about soluble complexes. It gains some support from the fact that the plasma of young puppies does not produce the typical response in adult recipients. Not only have young animals had short immunological lives, but it is well known that their ability to produce antibodies is poor (Brambell, Hemmings, and Henderson, 1951).

Another immunological possibility is that the individual specificity of dog plasma represents an orthodox antigen-antibody system with the somewhat unorthodox qualification that all the antibodies are of the naturally-occurring type. On this basis the phenomenon would resemble in some respects the ABO system in man, but would require a larger number of antigen and antibody species. One can, in fact, draw a rather close parallel with the ABO system if one assumes that the antigen-like member of the system resides in the skin cells and the antibody-like member in the plasma. Coombs, Bedford and Rouillard (1956) have recently demonstrated the A and B agglutinogens in human epidermal cells. In addition, it is well known that the anti-A and anti-B agglutinins appear only slowly in the sera of the newborn (Race and Sanger, 1958a), a situation resembling the delayed appearance of activity in puppy plasma.

The real difficulty with this hypothesis lies, of course, in our ignorance of the origin of natural antibodies. Even the origin of the anti-A and anti-B agglutinins is still disputed (Wiener, 1951): one view

is that their occurrence is genetically determined; the other view that their formation is induced by exposure to A and B substances from the environment. As Race and Sanger (1958b) point out 'there are but few facts to spoil the debate'. If the latter view is the correct one, and the natural antibodies of the ABO system do require antigenic exposure for their development, the analogy with the dog plasma phenomenon breaks down, for it is difficult to conceive of sufficient specific environmental factors which could induce the formation of the large number of antibody-like substances required by the hypothesis. (This is in distinction to the antigen-antibody-complex hypothesis, where antigen is entirely of exogenous origin, and antibody forms in the usual way.) If, however, the former view is the correct one, and natural antibodies are genetically determined, it is necessary only to increase the genetic complexity of the system to account for the specificity of dog plasma. That is, dogs would have genetically determined antigen patterns, and genetically determined antibody patterns, both of a nature such that particular antibodies of one animal are directed against particular antigens of another just as they are in the ABO system. A modification of this idea is presented later.

Another possibility, perhaps less plausible but more intriguing, is that the non-autologous plasma effect in dogs is a manifestation of the unknown mechanism whereby an animal is able to recognize antigenic material, including homologous tissues, as being 'foreign'. Every theory of antibody formation implies, tacitly or otherwise, the existence of such a recognition mechanism. Until more is known about the mechanism of antibody formation, there is little worth in speculating

about the possible relation of plasma specificity to it. However, it is interesting to note how conveniently the plasma specificity can be interpreted in the terms of the old theory of antibody formation, first put forth by Ehrlich (as discussed by Landsteiner, 1936), and repeatedly revived and modified since then (Cushing and Campbell, 1957). This theory, in its ammended form, holds that an animal possesses sufficient diversity in its protein molecules to be fore-armed with antibody or antibody-like material before antigenic exposure. Exposure, by removal of antibody or by the action of antigen-antibody complexes or by some other feedback mechanism, accelerates the production of antibody. The theory is not now in vogue, both because of the inability to demonstrate many instances of natural antibodies and of the difficulty in conceiving of the required diversity in native proteins. The first drawback is not a serious one, however, for minute amounts of antibody, not necessarily even present in the serum, satisfy the theory. The second drawback is the one usually cited against the theory, but the recent calculation by Haurowitz (1957) that an animal may be able to form only 10^3 to 10^4 distinct antibody species suggests that the diversity in protein structure required by the theory is not so formidable as previously suspected. The real virtue of the theory lies in the fact that it does not require an animal to have the ability to manufacture a 'new' molecule of a composition or configuration dictated by the antigen.

To put the plasma specificity phenomenon in the terms of this theory, we can think of an animal's possessing some large molecules, for example the homograft antigens, which are not shared by many other members of the population, and which are at the same time complementary to one or

more of the antibody species present in the rest of the population. If these large molecules were present in the plasma, (Oudin, 1956a,b, and Dray and Young, 1958, Chapter I, have demonstrated substances in rabbit serum antigenic to other members of the species), then, according to the theory, initial exposure of an animal to non-autologous plasma would lead to an antigen-antibody union and its consequences (in dogs, histamine release). The same result could occur if these large molecules were present in the tissues; one would then be transferring the antibody species with non-autologous plasma. This same reasoning requires that all substances which are antigenic in a given animal should produce an antigen-antibody union on initial exposure. The possibility that this is so is suggested by the immediate effects of heterologous sera and some other foreign proteins. Instances where immediate reactions to antigen are absent may represent only our inability to detect such reactions with present methods.

This hypothesis is presented only as a remote but interesting possibility. No serious attempt has been made to give all the supporting and refuting arguments, for these would require chapters.

Before concluding the discussion of possible immunological interpretations of the plasma specificity, it is necessary to comment on the observation (Chapter III) that mature offspring are not immunologically tolerant to maternal non-autologous factor despite their exposure to it in the early post-natal period. This result might be construed as evidence against either of the last two hypotheses. It is entirely possible, however, that the transfused maternal factor represents the antibody-like member of the system, and that antigenic exposure does not occur. It is also possible, as pointed out in

Chapter III. that the maternal factor is transferred not across the placenta, but through the colostrum; if this is indeed the case, then exposure of the offspring may be too late to induce tolerance, for Billingham, Brent and Medawar (1956b) have shown that exposure later than the first few post-natal hours is often unsuccessful.

A genetic interpretation. The previous suggestions emphasized the immunological aspects of the plasma specificity phenomenon, although the possibility of a genetic basis was suggested. Under the present heading is considered a hypothesis which attributes the phenomenon to a genetic system without introducing any immunological mechanisms.

If one accepts the concept that the isoagglutinins of the ABO system are under the same genetic control as the agglutinogens, one has a system whereby a particular person always possesses, by virtue of his genes, antibody-like substances capable of specific chemical union with whatever antigen-like substances of the system that person lacks. The possibility that the plasma specificity phenomenon may resemble this system at a higher level of complexity has already been mentioned. However, since we have no very good evidence that the plasma specificity represents an immunological system, we may redraw the analogy with the ABO system, this time without extending the analogy so far. We would now conceive of the plasma specificity phenomenon (a) as resting on the same type of genetic basis as the ABO system, (b) as having the same specificity for chemical union as the ABO agglutinogens and agglutinins, and (c) as differing from the ABO system in that exposure of an animal to agglutinogen-like factors he lacks does not induce increased formation of his agglutinin-like factors.

The detailed mechanisms necessary to complete this hypothesis are matters for even greater conjecture, but continuing the modified analogy with the ABO system, we might think of tissue cells, particularly the mast cells, as having on their cell membranes the agglutinogen-like substances, and of plasma as containing the agglutinin-like substances. Exposure of tissue cells to non-autologous plasma might then result in union of membrane factors with plasma factors, followed by lysis of the membrane and, in the case of mast cells, by histamine release.

Both this hypothesis, and the earlier hypothesis from which it was modified, receive some support from the recent demonstration by Smithies and others that at least some serum proteins are under rigorous genetic control, as discussed in Chapter I. It might be argued that a genetic system would require far greater complexity than that exhibited either by the ABO system or by the serum protein systems to account for the degree of specificity of dog plasma. This is partly true, but it should be remembered that the individual specificity exhibited both by the homograft rejection phenomenon and by the overall pattern of the erythrocyte antigens is due to genetic systems. It should be remembered too that a large number of phenotypes can result from a rather simple genetic system. For example, if the specificity of dog plasma were governed by only four alleles at each of four loci, and if the same assumptions were made to calculate the probability of finding a compatible pair as were made in Chapter I with reference to the homograft phenomenon, this probability value would be $(\frac{1}{4C_2})^4$, that is, less than 0.001. This rather fanciful calculation is presented simply to

show that it is not necessary to postulate an inordinately complex genetic system to account for the high degree of specificity exhibited by dog plasma.

In summary, it has been postulated that the individual specificity of dog plasma may be a manifestation of (i) the presence of soluble antigen-antibody complexes in the plasma (ii) a genetically controlled antigen system with naturally-occurring antibodies (iii) the unknown mechanism whereby an antigen is recognized as being foreign, and (iv) a genetically controlled system of plasma factors capable of specific union with genetically controlled tissue factors. The experimental evidence presented in this thesis does not lend particular support to any one of these hypotheses. Many of the experiments were not designed to do so. Those that were, particularly the experiments with inbred dogs, produced results which, instead of simplifying the problem, made it more perplexing (and, fortunately, more interesting).

Two obvious experiments present themselves for further analysis of the problem. If the immunological interpretation (aside from the soluble antigen-antibody-complex theory) is correct, then it should be possible to enhance the response to non-autologous plasma by appropriate antigenic stimulation. Experiments along these lines are now in progress. If the genetic interpretation is the correct one, the response to non-autologous plasma should be absent among identical twins. These are not available in dogs, but experiments with human identical twins are being started. If the response is absent, the results will require statistical analysis, for the response is not uniformly present among unrelated human subjects. If it is present, however, the evidence

against a genetic basis in man will be conclusive. To say how useful results obtained in man will be in interpreting the phenomenon in dogs will require more work with both species.

B. The Physiological Significance

Although most of the experiments described in this thesis are interesting from a physiological point of view, the results of the transfusion experiments have particular significance in this respect.

The demonstration in the dog that non-autologous plasma is a relatively inefficient plasma volume expander, and that it sometimes produces serious transfusion reactions, permits control, or at least appreciation, of an unexpected variable in experiments involving transfusion. The importance of this point lies in the frequency with which transfusion is used, whether as a support for a failing animal or as an integral part of an experiment. To quote Rushmer and Smith (1959), 'this experimental procedure is one of the most common in cardiac physiology'.

The demonstration also opens to reevaluation some previous experiments on the circulation in which transfusions of non-autologous blood or reservoirs stocked with non-autologous blood have been used. This is particularly the case with experiments in which blood volume changes have been important. It has been observed repeatedly, for example, that large transfusions of blood or plasma into normal dogs cause a rapid loss of plasma from the intravascular space (Metcalf, 1944; Seavers and Price, 1949; Guyton, Batson and Smith, 1951; Potvin, 1951; Huggins, Smith and Seibert, 1956; Huggins, Deavers and Smith, 1958). This observation has led to the opinion that a regulatory

mechanism exists for rapidly readjusting to normal an increased blood volume (Huggins, Deavers and Smith, 1958; Guyton, 1956). The results described in Chapter V show, however, that at least part of the plasma loss observed by earlier workers was undoubtedly due to the reaction to non-autologous plasma, and not to a homeostatic mechanism.

Indeed, the finding (Table XII) that the plasma volume expansion produced by autologous plasma transfusion was still complete at least an hour after transfusion shows that the correction of hypervolemia is surprisingly slow. Although the recipients of autologous plasma were slightly hypovolemic at the time of transfusion, the size of the transfusion was sufficient to make them hypervolemic: both the plasma volume and the total plasma protein were reduced by the plasmapheresis required to obtain the plasma, but they had returned sufficiently toward normal by the time of transfusion that, assuming ideal expansion, the volume of the transfusion produced an increase of 31% and the protein of the transfusion an increase of 25% over the normal values for plasma volume and total plasma protein determined before the bleeding procedures. The actual measurements showed that 45 minutes after transfusion the plasma volume was 33% above normal and the total plasmaprotein 35% above normal (cell volumes had not changed). Although this observation sheds little light on the largely unknown mechanism of blood volume regulation, it does show that an increased blood volume returns to normal much more slowly than previously thought. It suggests, moreover, that the correction of hypervolemia is considerably slower than the correction (Huggins, Smith, Deavers and Overton, 1957) of hypovolemia.*

*A teleologist would not find this surprising.

Another result from the transfusion experiments which may have some physiological significance was the unexpected observation of an apparent increase in total plasma protein after autologous transfusion 26% greater than could be accounted for by the amount of protein administered (Table XII). This divergence from the predicted increase in total protein, although statistically significant ($p<0.05$) is sufficiently small relative to the total protein value to be the result of a systematic error in experimental method. If it is real, however, it represents a curious effect. One explanation might be an acceleration of the normal lymph circulation such that, for a short period after the transfusion, normal protein-rich lymph enters the intravascular space while a filtrate unusually poor in protein leaves the capillaries. A more interesting possibility involves the concept of 'extra' plasma. It is reasonably well established that the total body hematocrit is considerably smaller than the large vessel hematocrit, particularly in splenectomized dogs (Reeve, Gregersen, Allen and Sear, 1953), suggesting that some of the plasma in small vessels is cell-free or cell-poor. It is not known whether all of this extra plasma is circulating or whether a portion of it is trapped (Deavers, Huggins and Smith, 1957). If the latter is the case, then it is possible that as the intravascular space expands to accommodate a transfusion some of the sequestered plasma becomes free and mixes with the circulating plasma. This would increase both the circulating plasma volume and the total circulating plasma protein. If at the same time the blood volume expansion were causing an increased rate of capillary filtration of protein-poor fluid, the net effect of transfusion could easily be an increase in total plasma

protein greater than the amount transfused without an equivalent increase in plasma volume. This was the situation encountered after the autologous plasma transfusions, although there is no way of knowing whether it was, in fact, due to the above mechanism. However, since the concept of trapped plasma is important but unsettled, it should be profitable to reexamine the question experimentally following the lead provided by the transfusion experiments.

C. The Clinical Significance

The observations on human subjects reported here may have considerable practical as well as theoretical interest: if the systemic response to non-autologous plasma parallels the cutaneous response, as it does in the dog, it is possible that a plasma incompatibility is responsible for some unexplained (and, more important, unrecognized) reactions to blood transfusion in man. The commonest (Weil, 1959) untoward reaction to blood transfusion now seen in clinical practice is the so-called allergic reaction, characterized by erythema, urticaria, and, rarely, by frank anaphylactoid manifestations. Although the allergic reaction is usually regarded as bothersome but harmless, its significance deserves reassessment in terms of associated changes in blood volume. If in man non-autologous plasma can produce not just urticaria, but, as in the dog, an inefficient expansion of the blood volume, then the situation becomes a serious one, for often the purpose of a transfusion is not to supply cells, but to increase the blood volume.

Transfusion experiments on normal human volunteers have recently been started by Drs. Burgen, Freedman and Hutchison, at the Montreal

General Hospital. The experimental design and procedure are rather similar to those of Group I of Chapter V. Of more importance is the fact that the results are also rather similar. It is not appropriate to describe these experiments in detail here; but it is interesting to note from the preliminary results that six autologous plasma transfusions have all produced efficient volume expansion and no untoward reactions, while seven of twelve non-autologous transfusions (obtained from donors of the same major blood groups) have resulted in urticaria (Figures XI and XII), and poor volume expansion.

On the basis of these results, it is tempting to speculate that the individual specificity of plasma, although more interesting in the dog from a theoretical standpoint, may have some clinical importance as well.

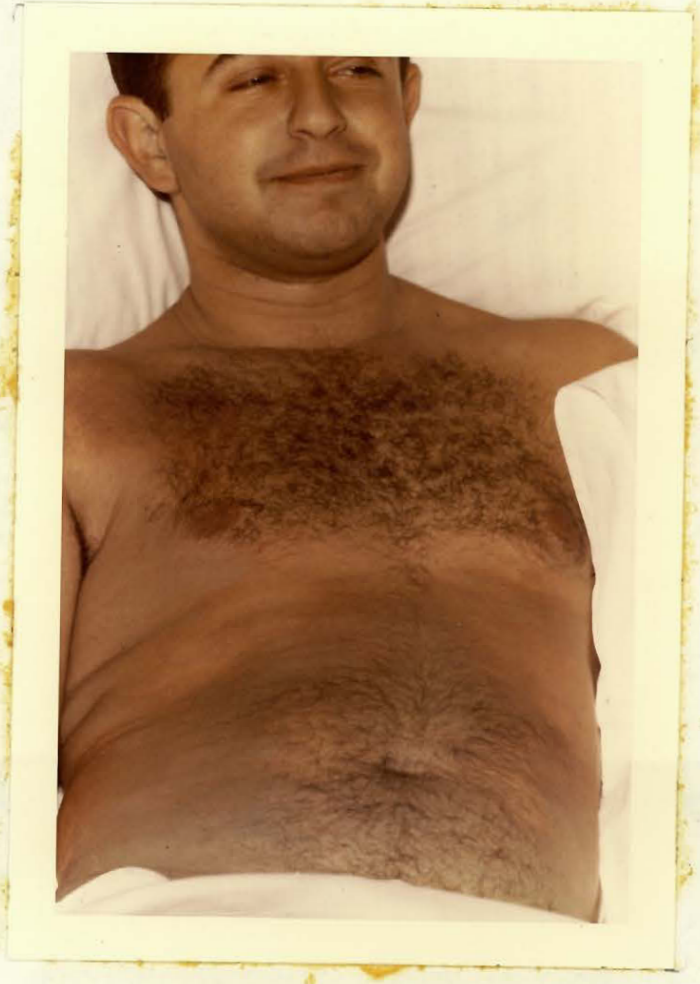


FIGURE XI - Urticarial Reaction to Non-Autologous Plasma Transfusion.

The photograph was taken immediately after the transfusion of 490 ml. of citrated non-autologous plasma. The subject had received 50 mg. of T-1824 intravenously one hour earlier, at the start of the transfusion. The areas coloured blue represent giant confluent wheals.

This subject had no untoward reaction to transfusion of the same volume of his own plasma two weeks earlier.

(Photograph by Dr. S. Freedman.)

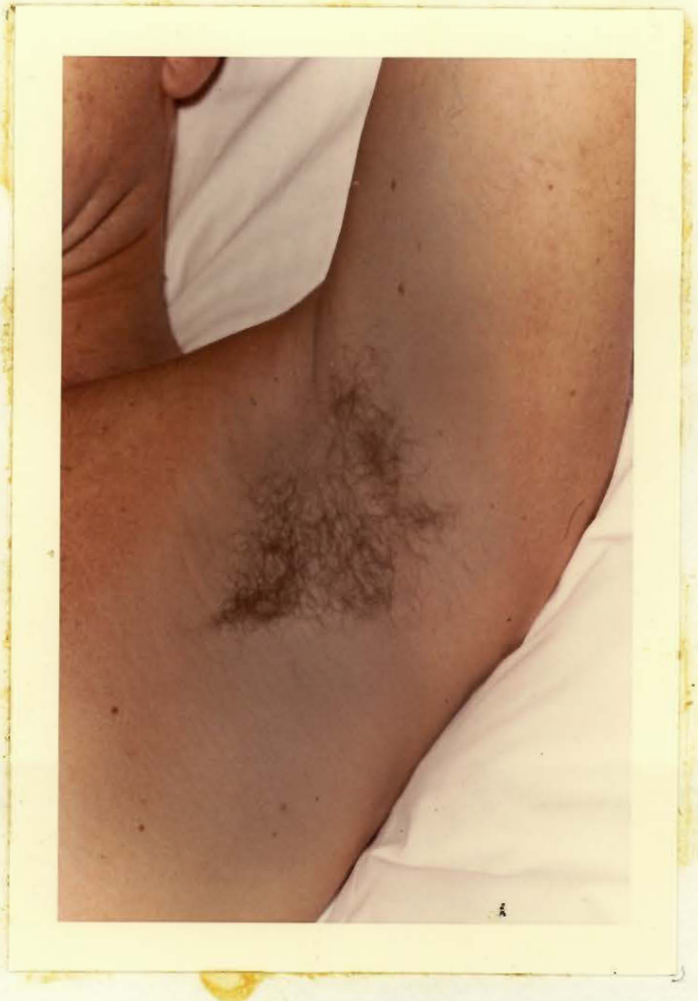


FIGURE XII - Urticarial Reaction to Non-Autologous Plasma Transfusion.

This subject received 440 ml. of non-autologous plasma. The experimental procedure was the same as that described for Figure XI. The blue areas again represent giant wheals. The redness of the chest and neck is true erythema, not photographic artefact. The subject had no untoward reaction to transfusion of his own plasma.

(Photograph by Dr. S. Freedman.)

SUMMARY

1. Dog plasma has been shown to have an extreme degree of individual specificity.

Among adult mongrel dogs, intracutaneous injection into one dog of plasma taken from another (non-autologous plasma) always caused a local increase in capillary permeability and a distinct wheal, while similar injection of the dog's own plasma (autologous plasma) never had this effect.

Dogs of standard pure breeds, including inbred dogs closely related genetically, showed the same phenomenon without exception.

2. Plasma taken from puppies during the second, third, and fourth post-natal weeks often failed to produce typical cutaneous responses in adult recipients. Activity rapidly increased to the adult level after the fourth week.

Plasma taken from puppies during the first post-natal day produced typical responses in an unrelated adult recipient, but no response in the mother, suggesting a transfer of activity from the mother. This transfer is ineffective in making offspring tolerant to maternal plasma, for mature offspring showed normal whealing responses to maternal plasma.

3. The individual specificity of dog plasma also revealed itself on intravenous injection.

Transfusions of non-autologous plasma, 20 ml./kg., nearly always produced minor to moderate urticarial reactions, and occasionally major reactions of an anaphylactoid type. The

increments in plasma volume and total plasma protein were equal on the average to only about half the amounts transfused.

Transfusions of autologous plasma under identical conditions never produced untoward reactions. The increments in plasma volume and total plasma protein were more than double those produced by non-autologous plasma transfusions.

No evidence was obtained to connect the reactions to non-autologous plasma with erythrocyte isoagglutinins.

4. In the dog, most if not all of the effects peculiar to non-autologous plasma were shown to be due to a release of endogenous histamine.

The wheaing response to intracutaneously injected non-autologous plasma was inhibited by the antihistamine mepyramine and was reduced in areas of skin previously exposed to a histamine liberator. More free histamine was recovered from skin injected with non-autologous plasma than from skin injected with autologous plasma. Marked increases in gastric acid secretion often followed non-autologous plasma transfusions. Mepyramine abolished the urticarial reactions to such transfusions, and improved the efficiency of non-autologous plasma as a plasma volume and protein expander.

5. Skin-testing of human subjects suggested that the specificity of dog plasma may be paralleled by a similar phenomenon in man.

6. Some mechanisms are postulated which might account for the individual specificity of plasma.

7. The general, physiological, and clinical significance of the results is discussed.

Note: The above summary outlines the original work contained in this thesis.

APPENDIX

PRELIMINARY EXPERIMENTS WITH SERUM FRACTIONS

No evidence has been presented so far concerning the physico-chemical nature of the factor(s) in dog plasma responsible for its individual specificity, although it was often implied in the thesis that this factor probably represents a large molecule. Some experiments have been performed in an attempt to determine what serum fraction contains the non-autologous activity. Most of the results are too preliminary to warrant their inclusion in the main body of the thesis; but it may be worthwhile to describe briefly some of these experiments, if only to show that this aspect of the problem is being considered.

All the experiments had the same general plan. Serum was used instead of plasma, since it is easier to handle during long experiments. Serum obtained from a donor dog was subjected to the particular experimental treatment (e.g. dialysis), after which it or its fractions were tested in the skin both of the donor dog and of a recipient dog. The importance of testing in the donor dog cannot be overemphasized, for physical or chemical manipulation of the serum frequently activated non-specific factors able to cause blueing on intracutaneous injection. The effect of a particular treatment on non-autologous activity was assessed in the usual way by comparing the responses to serial dilutions of untreated and treated serum.

Using this procedure, it was relatively easy to show that the

factor responsible for non-autologous activity is (a) non-dialysable, (b) stable at 60°C., and (c) partially precipitated by 19% Na_2SO_4. Serum dialysed at 4°C. against isotonic saline across cellophane for as long as three days always retained its full titre of non-autologous activity. Full activity was also retained by serum held in a water bath at 60°C. for one hour. Addition of 26.6% Na_2SO_4 to a final concentration of 19%, followed by incubation at 37°C. for one hour and then by centrifugation at 20,000 g for 30 minutes produced a precipitate which usually contained from 50 to 80% of the non-autologous activity present in the original aliquot of serum. These percentages were obtained by the skin-testing of a dialysed solution prepared by dissolving the precipitate in isotonic saline adjusted to pH 7.4 with phosphate buffer. Higher yields of non-autologous activity could not be obtained by increasing the final concentration of Na_2SO_4.

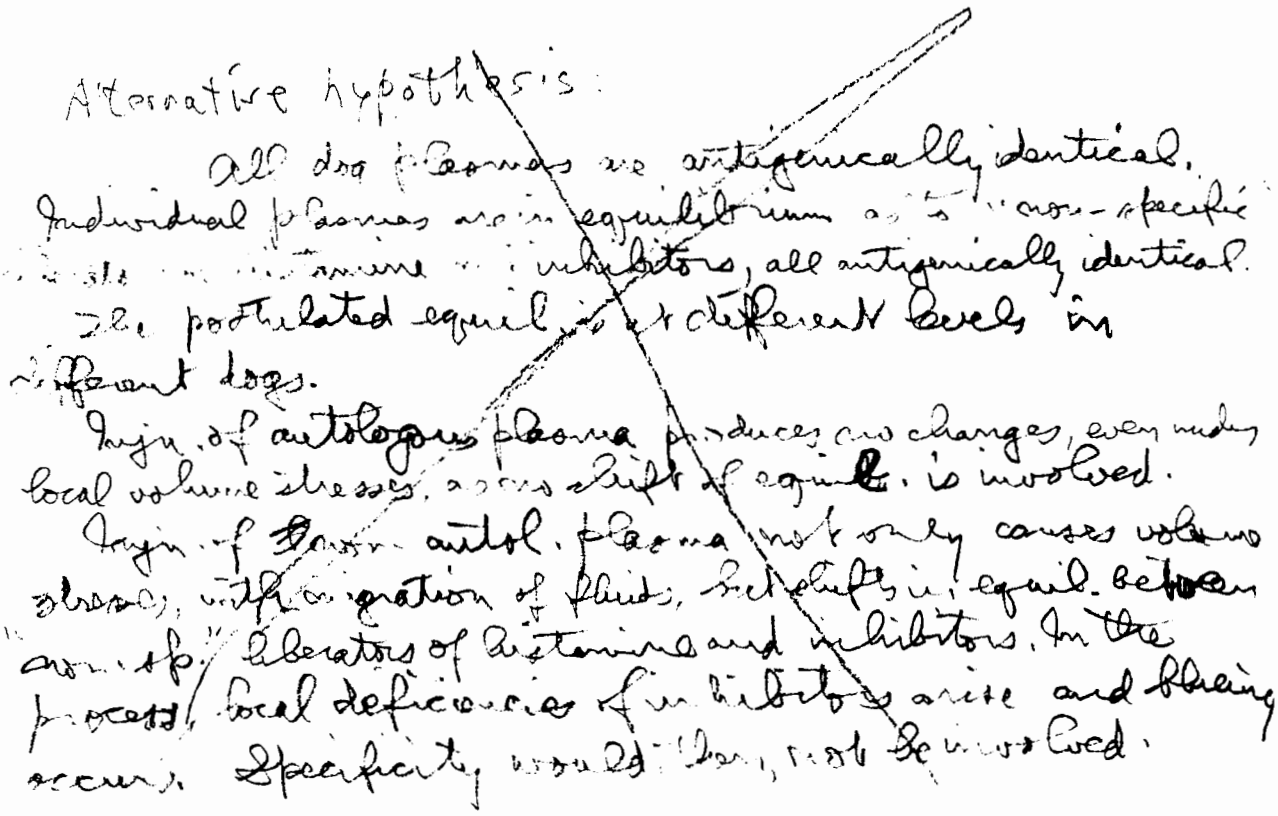
None of these procedures was complicated by the appearance of interfering permeability factors. However, more drastic treatment of the serum - for example ether extraction or trichloroacetic acid precipitation - always activated non-specific factors which caused marked cutaneous blueing whether the skin tests were performed on the donor or on the recipient dog. These interfering factors severely limited further exploration of the problem. However, the information at hand supported the working hypothesis that the serum fraction responsible for non-autologous activity is almost certainly of large molecular size, is probably a protein, and is possibly associated with the beta- or gamma-globulins.

An attempt was made to examine this hypothesis by the testing of

serum protein fractions obtained by Porath's method of preparative electrophoresis in a vertical cellulose column (Porath, 1956). Electrophoresis in phosphate buffer, pH 7.4, $\mu = 0.05$, produced four fractions which by paper electrophoresis had the characteristics of albumin + $alpha_1$-globulin, $alpha_2$-globulin, beta-globulin, and gamma-globulin. Skin-testing of these fractions gave rather disappointing results. Although the albumin and alpha-globulins had no activity of any kind, an interfering non-specific factor was present in both the beta- and gamma-globulin fractions. This factor produced so much blueing on intracutaneous injection that any evidence of non-autologous activity was completely obscured. A search was then made for means either to inhibit the effect of this factor or to prevent its formation. This effort finally met with some success: addition of small amounts of soya bean trypsin inhibitor (Worthington) to the fractions before testing inhibited much of the non-specific activity, but did not affect non-autologous activity; and electrophoresis in veronal buffer at pH 8.6 partially inhibited the appearance of non-specific activity.

Only a few further experiments have so far been performed with the improvements afforded by this observation. Electrophoresis in veronal buffer, pH 8.6, $\mu = 0.05$, produced good separation of six fractions which by paper electrophoresis had the characteristics of albumin, $alpha_1$-globulin, $alpha_2$-globulin, fast beta-globulin, slow beta-globulin, and gamma-globulin. Non-autologous activity appeared only in the latter two fractions; it was stronger in the slow beta than in the gamma fraction. However, the interpretation of these results was still complicated by the presence of some non-specific

activity, and by some technical difficulties. Definite conclusions must await further experiments.



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