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THE EFFECTS OF ADRENERGIC AGENTS ON THE HUMORAL IMMUNE RESPONSE

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CHAPTER I

INTRODUCTION

Adenosine 3', 5'-phosphate (cyclic AMP) has been shown to occupy a central position in biological control as the intracellular mediator of a variety of hormones. It has been detected in almost all cells studied to date and is involved in a great number of cellular processes. Recently, an altered response in one of these hormone systems has been linked to a malfunction of the cyclic AMP mechanism. The cause of this malfunction is thought to be a reduced response by the enzyme adenyl cyclase to the catecholamines released by the autonomic nervous system in reaction to stimuli. The result of this decreased adenyl cyclase activity is an unbalanced response by the autonomic nervous system in which alpha receptor mediated responses are dominant.

Szentivanyi has proposed that this unbalanced response by the autonomic nervous system is the fundamental abnormality in atopic bronchial asthma and, in fact, all atopic allergies. Considerable clinical data, mostly on the physiological and metabolic responses of individuals with bronchial asthma, seem to support this theory. Experimentally, many of these same responses can be produced in normal

individuals and animals by the use of agents which block adenyl cyclase.

A characteristic of the atopic allergies is the production of large amounts of reaginic antibody to a variety of substances which do not commonly immunize the general population. The role of the adenyl cyclase-cyclic AMP system in the immune response is as yet unknown. Both adenyl cyclase (the beta receptor) and the as yet unidentified alpha receptor are present in lymphoid cells. Experimental data suggest that these receptors may be important in both metabolic and functional processes of lymphoid tissues.

The purpose of this research was to examine the effects of agents involved in autonomic balance on immune responses in rabbits. The effects on the humoral immune responses, especially homocytotropic antibody production, of various compounds which increase or decrease cyclic AMP levels were determined. In addition, compounds (phenoxybenzamine, norepinephrine) which block or stimulate the alpha receptor were studied for these same effects.

LITERATURE REVIEW

The cyclic nucleotide, adenosine 3', 5'-monophosphate (cyclic AMP) was discovered by Sutherland and his co-workers during their study of the factors controlling the breakdown of glycogen in liver cells (1, 2). Epinephrine was found to increase the breakdown of glycogen. The action of epinephrine is mediated through its ability to activate a plasma membrane associated enzyme. This enzyme, later identified as adenyl cyclase (3), catalyzes the formation of cyclic AMP and inorganic phosphate from adenosine triphosphate (ATP). Further studies

showed this enzyme to be present in almost all animal cells, and to be activated by many hormones in addition to epinephrine (4).

As a result of their studies on the metabolic effects of the catecholamines in liver, muscle and adipose tissue, Robison et al. in 1967 (5) proposed that adenyl cyclase was the beta-adrenergic receptor. The concept of specific receptors on the cell which react with drugs such as epinephrine was originally postulated by Langley (6). Ahlquist (7) proposed the dual adrenoreceptor hypothesis on the basis of differences in the potency of sympathomimetic amines in producing responses in a variety of tissues, both in vivo and in vitro. Alpha receptor responses were usually involved with excitatory functions (vasoconstriction) and beta receptor responses with inhibitory functions (vasodilation and inhibition of uterine or bronchial musculature). The stimulating effects of the catacholamines on the alpha receptor were blocked by ergotoxine, dibenamine and tolzaoline.

Support for this theory was provided by Powell and Slater (8) who demonstrated that dichloroiso-prenaline (DCI) selectively blocked some inhibitory effects of epinephrine and isoproterenol on isolated guinea pig trachea. Similar observations for DCI were made by Moran and Perkins (9) who found that DCI blocked the positive inotropic effects in the isolated rabbit heart of small doses of epinephrine, norephinephrine and isoproterenol.

The beta receptors have now been further differentiated into beta-1 and beta-2 receptors. Beta-1 receptors are involved in lipolysis and cardiac stimulation, while beta-2 receptors mediate bronchodilation and vasodepression (10).

The identity of the alpha receptor is still uncertain. Belleau (11) has suggested that it may be part of a catecholamine catalyzed adenosine triphosphatase (ATPase) complex. This membrane bound complex may have as one of its functions the transport of calcium into the cell.

Hadden et al. (12) have demonstrated that noradrenaline enhances the uptake of both glucose and potassium in lymphocytes. This action is associated with a direct action on membrane ATPase (13).

Alpha adrenergic stimulation, then, is not associated with increased production of cyclic AMP and in certain tissues appears to decrease cyclic AMP levels (14, 15). Hadden et al. (16) suggest that this may be an indirect result of norepinephrine stimulation of ATPase, thereby reducing the amount of ATP substrate available for adenyl cyclase.

A number of other hormones have also been shown to increase cyclic AMP levels, including glucagon, adrenocorticotropic hormone, luteinizing hormone, vasopressin, thyroxine and melanocyte-stimulating hormone. Functions mediated through cyclic AMP include insulin release, synthesis of glucocortico steroids, thyrosine release, calcium and phosphate mobilization and release, synthesis of progesterone, water and ion movement, tachycardia and melanophore dispersion (17-19).

Sutherland et al. (17) formulated the two messenger hypothesis of hormone action. The hormone, as the first messenger which circulates in the blood, binds to the plasma membrane of the target cell and activates adenyl cyclase. Cyclic AMP, the second messenger, is

then formed on the inner surface of the cell membrane, diffuses through the cell and causes the physiological response.

It is to be expected that any malfunction of the adenyl cyclase-cyclic AMP mechanism would be reflected in an altered response by the cells involved.

Szentivanyi (20) has proposed that such a malfunction of the beta receptor, adenyl cyclase, is the fundamental abnormality in asthma, resulting in an excessive irritability of the bronchial tree. Relative insensitivity of the beta adrenergic receptors (beta blockade) is present in the bronchial glands, smooth muscle and mucosal blood vessels. With a diminished responsiveness of the bronchodilating mechanism mediated through the beta receptors, the constricting mechanisms mediated by the alpha receptors become dominant. This would account for the large variety of stimuli, both allergic and non-allergic, which can trigger bronchial constriction.

The beta-blockade theory offers an explanation for the many vagaries of atopy based on a single enzyme defect. In specific forms of atopy the receptor abnormality is postulated to involve the nasal mucosa, skin, antibody-forming cells and eosinophils, each independently and with varying degrees of severity. Considerable clinical experimental data supporting this theory have accumulated.

Middleton and Finke (21) report significantly less elevation of serum lactate, pyruvate and glucose levels in response to epinephrine injection in asthmatic patients than in normal subjects. Lockey *et al*. (22) noted that the intravenous infusion of epinephrine produced a smaller rise in blood glucose in asthmatic men. There was no

difference in changes in free fatty acids and glycerol levels or response to glucagon in normal and asthmatic individuals. Fireman et al. (23) found a diminished hyperglycemic response to epinephrine in asthmatic children and in a group of children with eczema.

Altered responsiveness to histamine has also been reported in atopic patients. Cole and Roberts (24), using a skin window technique, found that atopic subjects responded to the intradermal injection of histamine with the exudation of large numbers of polymorphonuclear leukocytes and with an eosinophilia of 3-15%. In non-atopic individuals, only an occasional cell was seen during the same period. The whealing response of atopic subjects was not different from that of the control group.

A few studies on cyclic AMP or adenyl cyclase levels in various tissues and fluids from atopic individuals have been reported. Bernstein et al. (25) found no increase in urinary cyclic AMP excretion by asthmatic children in response to subcutaneous epinephrine. Epinephrine induced a twofold increase in urinary cyclic AMP excretion in normal control subjects. Falliers et al. (26), in their study of discordant allergic manifestations in monozygotic twins, report lower adenyl cyclase activity and higher ATPase activity in leukocytes from the twin with asthma than the twin without asthma. Adenyl cyclase was not stimulated by isoproterenol in the cells of the twin with asthma. Logsdon et al. (27) also found that adenyl cyclase responsiveness to isoproterenol was absent in the leukocytes of asthmatics. Treatment with glucocorticosteroids restored adenyl cyclase responsiveness to isoproterenol.

Experimental support for the beta adrenergic theory has been provided by the use of beta adrenergic blocking drugs in animals and human volunteers. Beta adrenergic blockade has been shown to enhance bronchial reactivity to inhaled allergens (28) and to methacholine (29) in patients with seasonal allergic rhinitis without a previous history of bronchial asthma. An acute airway obstruction which seemed to reach the intensity of that found in patients with bronchial asthma was produced by progressively increasing doses of propranolol. McNeill and Ingram (30), using the whole body plethysmograph method, were able to demonstrate a 50 to 100 percent increase in airway resistance in normal human subjects receiving propranolol. Similar findings have been noted by Besterman and Friedlander (31).

Fishel et al. (32) found that pretreatment with Bordetella pertussis vaccine or dichloroisoproterenol significantly increased histamine and serotonin sensitivity in mice. Bordetella pertussis vaccine has been shown to have beta adrenergic blocking activity, as evidenced by its ability to lower adenyl cyclase activity and block responsiveness to epinephrine in mouse spleen cell suspensions (33). The glycemic abnormality associated with asthma can be reproduced in vitro (34) or in vivo (35).

The in vitro stimulation of leukocyte adenyl cyclase by adrenergic agents has been demonstrated with both broken cell (36) and intact cell (37) preparations. These studies have been extended to include the effects of cAMP and adrenergic agents on the metabolism and mitosis of lymphoid cells. Smith et al. (38-39) observed that dibutyryl-cAMP, isoproterenol, and theophylline inhibited by 60% the uptake of

tritiated thymidine by lymphocytes stimulated with phytohemagglutinin (PHA) when they were added during the first 24 hours of culture. No inhibition occurred when the agents were added after 24 hours of culture. Middleton and Hadden (40) found that this inhibition did not occur in the presence of propranolol.

Hadden et al. (41-45) studied the effects of timed pulses of various combinations of the catecholamines, cAMP, hydrocortisone, phentolamine and propranolol on tritiated thymidine uptake, glucose uptake, glycogen content, and lactate and $^{14}CO_2$ production in phytohemagglutinin-stimulated lymphocytes. Beta receptor stimulation by isoproterenol or high concentrations of cAMP inhibited thymidine uptake when they were added to cultures at the onset of incubation. However, when it was added after 72 hours of culture, cyclic AMP stimulated DNA synthesis. This effect may be the result of the activation of glycogenolysis by cAMP, thereby impairing the accumulation of glycogen needed for DNA synthesis which normally reaches peak rates after 72 hours of culture. Beta receptor stimulation at this time would enhance glycogenolysis and, thus, DNA synthesis.

Alpha receptor stimulation by norepinephrine could be shown to augment the PHA-induced tritiated thymidine uptake at both the onset and the end of incubation (43). Glucose uptake and catabolism to lactate were enhanced without a significant effect on glycogen content or $^{14}CO_2$ production. Enhanced DNA synthesis in response to alpha receptor stimulation was observed only in the presence of hydrocortisone.

Norepinephrine was found to stimulate lymphocyte plasma membrane ATPase activity through an alpha receptor mechanism (44). The

relationship of alpha adrenergic modulation of ATPase activity to glucose uptake is unknown at this time but may be explained by the cells' requirement to regenerate ATP. An alternate role could be the participation of ATPase in the transport of glucose across cell membranes.

Epinephrine inhibits mitotic activity in short-term cultures of peripheral blood mononuclear leukocytes stimulated by phytohemagglutinin when it is added at the beginning of culture (46). No effect was noted when epinephrine was added for only the final 24 hours of culture. Cyclic AMP stimulates mitosis of thymocytes (47, 48).

The involvement of the autonomic nervous system in the immune response has been suggested by Kokas et al. (49). They noted that stimulation of the splanchnic nerve in the dog increased the phagocytosis-enhancing ability of its serum for Staphylococcus aureus and Bacillus typhosus (Salmonella typhosa). This effect could be abolished by ligation of the adrenal veins and increased by the injection of adrenalin. Desoxycorticosterone or an extract of adrenal cortex had no effect in vitro. They concluded that the substances which produced the effect acted partly as opsonins and partly by direct stimulation of the leukocytes.

Draskoci and Jankovic (50) found that rats treated with reserpine had impaired cellular and humoral immune responses to bovine serum albumin, as well as thymic atrophy. The mechanism of action of this drug has not been fully established, but it has been shown to lower serotonin and catecholamine levels in various tissue (51).

Epinephrine given subcutaneously with diphtheria toxoid was found to enhance antibody production twelvefold in the guinea pig (52).

This adjuvant effect was postulated to be due to local vascular effects, since drugs which did not possess sympathomimetic activity had no adjuvant action. No enhancement of the immune response to bovine gamma globulin was observed in rabbits when the antigen was given intravenously with epinephrine (53). The data suggest, instead, that antibody production by the animals receiving epinephrine was suppressed.

Benner et al. (54) found that epinephrine reduced antibody production to egg albumin tenfold in rats when it was given at a dosage of 600 micrograms/kilogram every six hours for either five days before or ten days after antigen injection. A decline in antibody production to dinitrophenyl-egg albumin has also been reported in mice treated with epinephrine after immunization (55), however the difference between experimental and control values was not statistically significant. Epinephrine added to the culture fluid of spleen fragments of mice immunized with sheep red blood cells has been observed to suppress the number of plaque forming cells (56). The addition of epinephrine for a 24 hour period on day three, four or five of culture produced no reduction in plaque forming colonies (57).

Isoproterenol, another beta receptor stimulator, was found to suppress both total and homocytotropic antibody formation to dinitrophenyl-egg albumin (55). Pearlman (58), however, reported an enhancement of the in vitro response by mouse spleen cells to sheep red blood cells when isoproterenol or aminophylline in concentrations of 10^{-4} M to 10^{-7} M was added to the cell cultures.

Ishizuka et al. (59) found that cAMP and dibutyryl adenosine 3', 5'-cyclic phosphate (dib-cAMP) enhanced antibody formation when

they were injected with sheep red blood cells or added *in vitro* to a spleen cell suspension. The administration of cAMP 24 hours after antigen produced even greater stimulation. Theophylline, a stabilizer of cAMP was found to enhance the stimulatory effects of poly A:U (60). This effect was particularly pronounced with concentrations of poly A:U which are normally inactive. Theophylline alone did not alter the immune response.

Alpha receptor stimulation by norepinephrine at concentrations of 10^{-4} M to 10^{-7} M enhanced the *in vitro* antibody response of mouse spleen cells to sheep red blood cells up to fivefold at the highest concentration (56). Makino and Reed (57) found, however, that norepinephrine at a concentration of 9×10^{-5} M inhibited the antibody response to sheep red blood cells.

Conflicting reports have also appeared regarding the effects of alpha adrenergic blockade. Pieroni and Levine (61) observed that dibenzyline, a haloalkylamine alpha adrenergic blocking agent, suppressed the immune response of mice to tetanus toxoid injected with or without pertussis vaccine. Each mouse received one milligram of dibenzyline. The weights of the mice were not given, but assuming an average weight of 25 grams, this would represent a dose of approximately 40 mg/kg. Hadden (62) reported that doses of 0.2 to 0.5 mg/kg (the human therapeutic equivalent) had no effect on the cellular and humoral immune response in the rabbit. Similar observations were made by Rosenblatt and Johnson (53) who used a dose of 3 mg/kg.

Beta adrenergic blockade has been reported to exert an adjuvant effect on antibody production (54). Butoxamine and propranolol,

at dosages of 15 mg/kg and 25 mg/kg, respectively, when administered every six hours for ten days beginning immediately after the injection of egg albumin, increase antibody production threefold. When the drugs were given at six hour intervals for five days before the antigen, butoxamine had little effect and propranolol decreased antibody production tenfold.

In 1921, Prausnitz and Kustner (63) demonstrated in an allergic individual a serum factor which caused a local sensitization of the wheal and erythema type in the skin of a normal human subject. This factor was later detected by Coca and Grove (64) in the serum of subjects with hay-fever and asthma and was termed by them atopic reagin. These authors showed that the local passive sensitization lasted for four weeks or longer and that the skin-sensitizing capacity of reagin was diminished by heating the serum for one-half hour at 56^{o} C.

During the next 40 years reaginic antibodies were studied by numerous investigators, however their exact nature remained unknown until the discovery of a new immunoglobulin class by Ishizaka et al. (65) and Johansson and Bennich (66). This immunoglobulin class has been designated IgE (67).

IgE is a gamma globulin with a sedimentation coefficient of 8.25 and a molecular weight of 200,000 (68, 69). The mean normal adult serum concentration is approximately 250 nanograms per milliliter. A special characteristic of this immunoglobulin is its ability to fix to the surfaces of tissue mast cells and circulating basophils. The union of antigen with specific cell-bound IgE antibody causes the cell to release histamine and other vasoactive materials responsible

for the edema and vasodilation associated with immediate type allergic reactions (70).

Increased amounts of IgE in the serum have been found in the atopic allergies, including asthma (71), eczema and seasonal rhinitis (72-73), allergic aspergillosis (74), food hypersensitivity and parasite infections (75-77). High serum levels have also been reported in patients who have experienced anaphylactic reactions (78) as well as some non-atopic diseases including pulmonary hemosiderosis, severe liver disease (72) and IgE multiple myeloma (66).

While much of the data support the thesis that IgE is involved with allergic disease, some investigators have suggested that the immunoglobulin might also have a protective role. Callerame et al. (79), based on their study of bronchial biopsies from asthmatics, reported large numbers of IgE-containing cells in tissue showing inflammation, suggesting that this immunoglobulin plays a normal part in the inflammatory response. Ammann et al. (80) report data suggesting that a deficiency of IgE may be related to recurrent respiratory tract infection. The physicochemical properties of IgE and its role in the mechanisms of reaginic hypersensitivity have been reviewed by Ishizaka and Ishizaka (81), and Ishizaka (82), and more recently were the subject of a symposium (83).

Initially it was thought that antibodies capable of sensitizing the skin of the homologous species for passive cutaneous anaphylaxis were rare in species other than man. They have now been found in almost all animals studied (84). These antibodies have now been designated as homocytotropic antibodies because of their ability

to fix to cells of animals of the same species in which they were formed or of closely related species (85).

The production of high levels of homocytotropic antibody in animals, except in the case of parasite infections (86) and a few cases of spontaneous allergy of the P-K type in dogs and cattle (87-88), normally requires the administration of antigen in combination with some form of adjuvant. A variety of substances have been used, including incomplete and complete Freund's adjuvant (89), aluminum hydroxide (90), and a number of bacterial vaccines including Bacillus subtilis (91), Corynebacterium parvum (92), and Bordetella pertussis (93).

B. pertussis vaccine is of special interest because it appears to have pharmacological properties associated with beta adrenergic blocking agents (51, 94-101). Finger et al. (102) present evidence suggesting that B. pertussis causes multiplication of antigen sensitive target cells or affects the initial stages of differentiation of these cells. A similar conclusion was reached by Reed et al. (55). The cellular immune response may be decreased by B. pertussis administration (103).

The wide use of the rabbit in immunological research and its reputation as a good antibody producer have prompted considerable study on homocytotropic antibody in this species.

The first report of an antibody capable of sensitizing rabbit skin for homologous passive cutaneous anaphylaxis (PCA) was that by Zvaifler and Becker (104). The antibody was produced by immunization with bovine gamma globulin coupled with dinitrophenyl sulfonic

acid sodium salt in Freund's complete adjuvant. Activity was detected as early as the sixth or seventh day following immunization and was gone by the third week. The antibody was heat- and mercaptoethanol-labile, non-precipitating, non-complement fixing, required a three day latent period for skin fixation to demonstrate PCA activity, had an electrophoretic mobility faster than rabbit IgG globulin and was eluted from Sephadex G-200 between IgM and IgG.

A rabbit antibody with skin-sensitizing activity was also reported by Onoue et al. (105). They concluded that rabbit PCA activity was found in the 7S IgA globulin fraction.

Zvaifler et al. (106) observed that rabbits infected with Schistosoma mansoni produced a heat- and mercaptoethanol-labile PCA antibody seven to twelve weeks after infection. Antibody titers remained elevated for three to six weeks, then declined to low levels or disappeared completely. Reinfection at 36 weeks caused a return of the antibody in half of the animals.

Lindqvist (107) reported the presence of a heat stable, mercaptoethanol sensitive PCA antibody with an estimated molecular weight of 200,000 in rabbits immunized with tetanus toxoid. The antibody was detectable in serum over a period of 6 to 133 days after a primary immunization. Its appearance was not affected by changes in the route of immunization, treatment with 6-mercaptopurine or splenectomy (108).

Revoltella and Ovary (109) reported that the use of aluminum hydroxide as an adjuvant resulted in the formation of homocytotropic antibody in all of their rabbits, in contrast to the variable results obtained with complete Freund's adjuvant. With small amounts of

antigen it was possible to obtain homocytotropic antibody in only three out of ten animals. Similar findings were noted by Freeman et al. (90). Kindt and Todd (110) could find no significant difference between homocytotropic antibodies produced in the primary and the secondary immune response.

Heat-sensitive and heat-resistant homocytotropic antibodies have been found in the same serum from rabbits immunized with soluble crude Ascaris extracts, or with extracts absorbed onto aluminum hydroxide (111). Both antibodies were inactivated by reduction and alkylation.

Oral administration of egg albumin or bovine serum albumin resulted in the production of homocytotropic antibody by a small number of rabbits (112). Serum titers of anti-bovine serum albumin homocytotropic antibody persisted for several months following the cessation of antigen administration.

An enhancement of homocytotropic antibody formation by the administration of 19S antibodies one day before or after antigenic stimulation was reported by Strannegard and Belin (113). The administration of 7S antibody prior to immunization, or together with the antigen, resulted in the inhibition of homocytotropic antibody formation (114-115).

Pinckard et al. (116) found that neonatal rabbits immunized with alum-precipitated or soluble bovine serum albumin (BSA) in conjunction with Corynebacterium parvum adjuvant produced only homocytotropic antibody during the neonatal period and throughout adult life. When they were immunized at seven days of age with soluble BSA, the

animals became immunologically unresponsive to BSA, with the exception of anti-BSA homocytotropic antibody produced when they were adults. Immunization on day seven with BSA and C. parvum resulted in the production of anti-BSA antibody detectable by all the test procedures.

Henson and Cochrane (89) showed that rabbits immunized with BSA and complete Freund's adjuvant produced both heat labile and complement-, platelet- and neutrophile-dependent homocytotropic antibodies. The complement-dependent antibody persisted in the circulation for long periods of time and had physicochemical characteristics suggesting a rabbit IgG antibody.

Characterization of the physicochemical and biological properties of homocytotropic antibody against dinitrophenyl-bovine gamma globulin and egg albumin has been performed by two groups of investigators (117-118). Neither group was able to demonstrate a complement-dependent, heat stable homocytotropic antibody. The homocytotropic antibody found in both studies was a beta globulin with a sedimentation coefficient of approximately 8.5S which eluted from a Sephadex G-200 column earlier than IgG. The molecular weight was estimated to be approximately 200,000. The antibodies were heat labile and required a skin fixation period of 72 hours. The ability of the antibody to sensitize skin for the homologous PCA reaction was not blocked by absorption with antisera specific for rabbit immunoglobulins G, A or M.

Because the unique physicochemical properties and biologic function of this immunoglobulin are so similar to human IgE, Ishizaka et al. (118) have tentatively designated it rabbit IgE. The demonstrated ability of the rabbit to produce a homocytotropic antibody similar

to human immunoglobulin E makes it an ideal model for studying the effects of adrenergic agents on the immune response.

The purpose of this research was to examine the effects of agents involved in autonomic balance on the humoral immune responses, especially homocytotropic antibody production. Various compounds which increase or decrease cyclic AMP levels and compounds (phenoxybenzamine, norepinephrine) which block or stimulate the alpha receptor were studied for these same effects.

CHAPTER II

MATERIALS AND METHODS

Experimental Animals

Rabbits of both sexes and of mixed breeds, weighing approximately 1.2 - 3.4 kg, were used in these experiments. All of the animals were obtained from local sources and fed a commercial rabbit chow and water ad libium. The animals were housed in individual cages or in groups of five in large hutches. All animals were observed for at least 72 hours before being used for experimental purposes.

Preparation of Antigen and Adrenergic Agents

The following agents were dissolved in pyrogen-free 0.85% NaCl (McGraw Laboratories, Glendale, California) in the indicated concentrations: egg albumin (5x crystallized, B-grade, Calbiochem, San Diego, California), 0.0001, 0.001, 0.01, 1.0, 10, 20, 100, and 200 mg/ml; bovine serum albumin (Armour Pharmaceutical Company, Kankakee, Illinois) in concentrations of 0.0001, 0.001, 0.01, 0.1, 1, 10, 20 and 100 mg/ml; norepinephrine (Arterenol, Sigma Chemical Company, St. Louis, Missouri), 600 micrograms/ml; propranolol (furnished through the courtesy of Dr. R. O. Davies, Ayerst Laboratories, New York, N. Y.) 2, 20, and 200 mg per ml; Sotalol (furnished through the courtesy of Dr. G. R. McKinney, Mead Johnson & Company, Evansville, Indiana), 200

mg per ml; DL-isoproterenol (Sigma Chemical Company, St. Louis, Missouri), 250 micrograms per ml. Phenoxybenzamine (Dibenzyline, furnished through the courtesy of Dr. G. J. Oswald, Smith, Kline, and French Laboratories, Philadelphia, Pennsylvania) was dissolved in absolute ethyl alcohol (reagent grade, U. S. Industrial Chemical Company, New York, N. Y.) in concentrations of 4, 40, 200, and 400 mg per ml.

Dibutyryl cyclic AMP (N^6O^2-dibutyryladenosine 3'-5' cyclic phosphate, Na, 8-hydrate, A-grade, Calbiochem, San Diego, California) was dissolved in cold pyrogen free 0.85% NaCl in a concentration of 20 mg/ml immediately prior to use and stored in ice during the immunization procedure.

Initial Studies

Rabbits were injected with propranolol, 10 mg/kg intravenously (I.V.) or 10, 15, 20, 25 and 30 mg/kg subcutaneously, to determine the maximum tolerated dosage for this drug.

Immunization of the Animals

Animals were immunized by intrafootpad injections of egg albumin, 25 mg per kg, either alone or mixed with one of the following agents: propranolol, 0.2, 2.0, and 20 mg per kg; Sotalol, 20 mg per kg; isoproterenol, 50 micrograms per kg; norepinephrine, 120 micrograms per kg; phenoxybenzamine, 0.4, 4.0, 20, and 40 mg per kg. The mixtures were made up to a volume of 1 ml by the addition of 0.85% NaCl. Each footpad was injected with 0.25 ml of the mixture. Certain animals received a secondary injection of antigen. This was given either as 4 mg per kg in 1 ml of 0.85% NaCl, 0.25 ml per footpad,

or as a single injection of 10 mg per kg in 1 ml of 0.85% NaCl intramuscularly in a hind leg. For some animals, this mixture also contained 20 mg per kg of propranolol, 75 micrograms/kg of isoproterenol, or 1 ml of absolute ethyl alcohol. In other animals the propranolol and isoproterenol were given in a separate 1 ml injection intramuscularly in the opposite leg.

Additional rabbits were injected with egg albumin, 25 mg/kg in 0.5 ml of 0.85% NaCl and 0.5 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). The egg albumin and the adjuvant were emulsified by homogenizing for 4 minutes on a Sorval Omnimixer (Ivan Sorvall, Inc., Norwalk, Connecticut).

In one experiment, dibutyryl cyclic AMP, 44 mg per kg in 4 ml of cold 0.85% NaCl, was injected into the footpads, 1 ml per footpad. Thirty minutes later, footpad injections of egg albumin, 25 mg/kg, alone, and mixed with 20 mg per kg of propranolol or 0.4 mg per kg of phenoxybenzamine were administered. In all cases, the second injection consisted of one ml total volume, 0.25 ml per footpad.

Collection and Storage of Serum

Prior to immunization, and at intervals thereafter, all animals were bled by cardiac puncture or from the central artery of the ear. The blood was allowed to clot at room temperature and was placed in a refrigerator at 4^{o} C overnight. The clot was then discarded and the serum centrifuged at 1000 G (3000 RPM) for five minutes. One to five ml samples of the cell-free serum were placed in screw cap vials and stored without chemical preservatives at -20^{o} C. Samples were

thawed as needed immediately before use and were then refrozen. In some instances the samples were kept for several days at 4° C after thawing.

Passive Cutaneous Anaphylaxis (PCA)

Homocytotropic antibody titers were determined by a slight modification of the method of Zvaifler and Becker (80). These assays were performed as follows:

Serum from immunized rabbits was diluted with pyrogen-free 0.85% NaCl to obtain serum concentrations of undiluted, 1/4, 1/16, 1/64, 1/128, 1/256, and in some cases 1/512 and 1/1024. For convenience, these concentrations will be referred to as their $-\log_2$ equivalents (0, 2, 4, 6, 7, 8, 9 and 10, respectively).

The backs of nonimmunized recipient rabbits were shaved with an electric clipper (John Oster Manufacturing Company, Milwaukee, Wisconsin). The shaved area was then marked with a water proof marking pen into four rows of squares, each row containing 7 squares approxmiately 40 x 40 mm each. Eighteen to 42 hours later, 0.15 ml of serum was injected intradermally into the center of each square with a 26 gauge needle and disposable tuberculin syringe (Becton, Dickinson and Company, Rutherford, New Jersey). A single syringe and needle were used for all the dilutions of a single serum. The most dilute sample was injected first and the undiluted serum last. Sufficient material was drawn into the syringe each time to allow the injection of two animals. An injection of 0.15 ml of the pyrogen-free, 0.85% NaCl used for serum dilutions was made into the skin of the nuchal region at the time of the serum injections as a negative

control.

Sixty-eight to 72 hours after the serum injections, 0.1 ml (0.55 mg) of histamine dihydrochloride (B grade, Calbiochem, Los Angeles, Calif.) was injected intradermally into a previously uninjected site on the back as a positive control. The animal then received 2.0 ml of egg albumin, 10 mg per ml, and 2.0 ml of 0.5% Evan's Blue dye (Warner-Lambert Company, Morris Plains, New Jersey) via the margional ear vein. After at least one hour, the animal was killed by cervical dislocation and skinned. With the aid of a light box, the underside of the skin was examined for areas of bluing. Measurements of the diameter of each area were made in two directions and recorded in millimeters. In some cases the area of bluing was recorded only as "slight."

All of the serum samples from a single experimental group were placed on the same recipient animal and each sample was tested in the skin of two animals.

Latent Period Required for Skin Fixation of Antibody

Rabbits were injected with serum as described under PCA, then challenged with egg albumin and Evan's Blue dye at 24, 48, or 72 hr.

Passive Hemagglutination (PHA)

A modification of the procedure of Johnson et al. (119) was used to couple egg albumin to rabbit red blood cells. Reagent grade chemicals (Fisher Scientific Company, Fairlawn, New Jersey) were used to prepare all solutions. A modified Alsevers solution was prepared

as follows: To a 1000 ml volumetric flask were added 20.5 gm D-glucose, 20.0 gm disodium ethylenediamine tetraacetate, 4.1 gm of NaCl, and 10 ml of normal rabbit serum. The contents were brought to volume with distilled water and the pH was adjusted to 7.0 using 1N NaOH or 1 N HCl.

A phosphate-buffered saline (PBS) solution, pH 7.2, was prepared by the addition of 24 ml of 0.15 M KH_2PO_4 and 76 ml of 0.15 M Na_2HPO_4 to 100 ml of 0.85% NaCl.

Normal rabbit blood was collected from the central artery of the ear into a syringe containing an equal volume of modified Alsevers solution. After mixing by slowly rotating the syringe, the blood was expressed into a conical tube and centrifuged for 5 minutes at 1000 G (3000 RPM) in an International model HN-S centrifuge equipped with a No. 215 horizontal head (International Equipment Company, Needham Heights, Mass.). The plasma and buffy layer were then removed by aspiration and discarded. Ten ml of modified Alsevers solution were added to the tube and suspension of the erythrocytes was accomplished by touching the tube to a vortex mixer (Scientific Industries Incorporated, Springfield, Massachusetts).

The erythrocytes were then packed again at 1000 G for 5 minutes. This washing procedure was repeated three times. After the last wash a volume of PBS equal to the packed cell volume was added to make a 50% (V/V) cell suspension.

To prepare 50 ml of a 0.5% suspension of egg albumin coupled red blood cells, 2.5 ml of 1-ethyl-3-(3-dimethylaminopropropyl) carbodiimide HCl, 100 mg/ml, (Ott Chemical Company, Muskegon, Michigan)

was added to 15 ml of egg albumin (10 mg/ml) in PBS and 0.5 ml of the 50% suspension of rabbit red blood cells. The mixture was allowed to stand for 60 minutes at room temperature with intermittent shaking. The cells were then washed once in PBS and 3 times in modified Alsevers solution. After the last wash, the cells were suspended to a volume of 50 ml. Sensitized cells remained stable for at least 7 days at 4^{o} C if they were washed 3 times with modified Alsevers solution just before use.

Titration of antibody was performed by the microtiter method. A 0.025 ml volume of modified Alsevers buffer was added to each well of a V-bottom, disposable plastic tray (Linbro Chemical Co. Inc., New Haven, Conn.) by means of a calibrated pipette dropper attached by plastic tubing to a serum bottle. A pipetting device, equipped with a disposable polyethylene tip (Carworth Division, Becton, Dickinson, New York, New York) was used to deliver 0.025 ml of serum into the first well in each row. Sera were diluted serially to either $1:2^{12}$ or $1:2^{24}$ with a multi-microdiluter equipped with micro diluters of 0.025 ml capacity (Cooke Engineering Company, Alexandria, Virginia). A 0.025 ml volume of sensitized red cells (0.05%) was then added to each well. Each sample was titrated in duplicate and each plate also contained a saline control and a normal serum control. The plates were incubated overnight at 4^{o} C. The greatest serum dilution in which the red cells did not form a compact button on the bottom of the well was recorded as the end point. When duplicate samples gave different end points, the average of the two values was recorded.

Mercaptoethanol Sensitivity of Homocytotropic and Agglutinating Antibodies

The effects of reduction with 2-mercaptoethanol followed by alkylation with iodoacetamide on homocytotropic and agglutinating antibody titers were examined. Rabbits were immunized with egg albumin, 25 mg/kg, and propranolol, 20 mg/kg, or phenoxybenzamine, 0.4 mg/kg, by intrafootpad injections. Sera from both early (7 day) and late (49 day) bleedings were tested.

The technique described by Zvaifler and Becker (80) was used. One ml samples of serum were placed in dialysis tubing (Fisher Scientific) and dialyzed against 500 ml of 0.1 M 2-mercaptoethanol for 3 hr at room temperature. They were then dialyzed against 0.02 M iodoacetamide (Calbiochem) for 4 hr at room temperature, followed by several changes of phosphate-buffered saline (PBS) for 18-24 hr at 4^{o} C. The PBS was prepared as follows, using reagent grade Fisher chemicals: 1 L of distilled water was added to 6.8 gm of NaCl, 1.483 gm of $Na_2HPO_4 \cdot 7\ H_2O$, and 0.433 gm of $KH_2PO_4 \cdot H_2O$. The pH was adjusted to 7.2 using 1N HCl or 1N NaOH. Control samples of serum were dialyzed against PBS instead of 2-mercaptoethanol, then treated with iodoacetamide and dialyzed against PBS in a manner identical to that used with the test samples. Sera were tested for PCA and PHA activity as described above.

Heat Stability of Homocytotropic and Agglutinating Antibodies

A two ml volume of whole serum was placed in a constant temperature water bath at 56^{o} C. Samples were removed at 1, 2, 3, and 4 hrs and tested for PCA and PHA activity. The interval between

completion of heating and injection for the PCA reaction did not exceed 2 hr, however samples were stored overnight at 4^{o} C before the PHA tests were performed. Sera from both early (7 day) and late (49 day) bleedings were examined for heat stability. The sera were from rabbits immunized with egg albumin, 25 mg/kg, and propranolol, 20 mg/kg or phenoxybenzamine, 0.4 mg/kg, by intrafootpad injections.

Titration of Skin Test Materials

The optimum concentrations of egg albumin, bovine serum albumin, and histamine for the direct skin test were determined. A rabbit producing homocytotropic antibody was shaved and injection sites were marked as described above. Dilutions of the antigen, egg albumin, and bovine serum albumin for specificity control, were made in 0.85% NaCl. The following concentrations were injected intradermally in a 0.10 ml volume: 10,000, 1,000, 100, 10, 1, 0.1, and 0.01 microgram. Histamine in 0.85% NaCl, containing 5,500, 550, 55, 5.5, 0.55, 0.055, and 0.0055 micrograms/0.10 ml, was also injected intradermally.

After completion of the intradermal injections, 2.0 ml of 0.5% Evans Blue dye was given intravenously. One hour later the areas of bluing were measured and recorded as described previously.

Effects of Propranolol on the Direct Skin Test and the Reaction to Histamine

A group of rabbits immunized three weeks previously with egg albumin, 25 mg/kg, and propranolol, 0.2, 2.0, and 20 mg/kg, were examined, by direct skin test, for their response to egg albumin, bovine serum albumin, histamine, and saline, before and after the injection of propranolol, 20 mg/kg. The concentrations of the test substances

were: (1) Egg albumin - 10 micrograms/0.1 ml, (2) Bovine serum albumin - 10 micrograms/0.1 ml, (3) Histamine - 0.55 micrograms/0.1 ml, and (4) Saline (0.85% NaCl). All dilutions were made in 0.85% NaCl. Each animal received 2.0 ml of 0.5% Evan's Blue dye prior to intradermal injection of the test materials. Forty-five to sixty minutes after application of the test materials the areas of bluing were observed through the skin and recorded as described above. Each animal then received 20 mg/kg of propranolol in a 1.0 ml volume of 0.85% NaCl, 0.25 ml being injected into each footpad. A second series of intradermal injections of the test materials were made 1/2 hour after the propranolol injection. Forty-five to sixty minutes after this series of injections the areas of bluing were recorded as described above.

Tests for Blocking Antibody

These procedures were developed to test the possibility some class of antibody might block immediate hypersensitivity by occupying tissue sites competitively. Sera, without homocytotropic antibody for egg albumin detectable by PCA, were tested for their ability to block the immediate skin reaction to egg albumin.

Serum samples, to be tested by this procedure, were selected from animals which had been immunized with egg albumin, 25 mg/kg, alone or in combination with one of the following drugs: (1) Sotalol, 20 mg/kg, (2) Norepinephrine, 120 micrograms/kg, (3) Isoproterenol, 50 micrograms/kg. Samples obtained from early (day 7), middle (day 28 and late (day 49) bleedings were assayed. Preimmunization samples were included as controls.

A sensitized rabbit, which had been immunized three weeks previously with egg albumin, 25 mg/kg, and propranolol, 20 mg/kg, by footpad injection, was prepared as described prevously for the PCA test. Serum samples, either undiluted or diluted 1:5 with pyrogen-free 0.85% NaCl were injected in 0.15 ml volumes into separate sites. Approximately 30 minutes after the serum injections, 2.0 ml of 0.5% Evan's Blue dye was injected I.V. Each site was then injected with 0.1 ml of egg albumin in 0.85% NaCl, 10 micrograms per 0.1 ml. One hour later the rabbit was killed and skinned and the areas of bluing on the underside of the skin were measured and recorded as described previously.

In a second experiment, a sensitized rabbit was injected intradermally with 0.15 ml volumes of sera from a rabbit immunized with egg albumin, 25 mg/kg, and isoproterenol, 50 micrograms/kg, by footpad injections. These sera were negative for homocytotropic antibody, as measured by the PCA test. The prepared skin sites, and normal skin sites, were challenged at 1/2, 6, 24 and 41 hours with a 0.1 ml (10 microgram) intradermal injection of egg albumin. Two ml of 0.5% Evan's Blue dye were injected intravenously prior to the 1/2 and 22 hr challenges. The areas of bluing were measured through the skin and recorded as described previously.

In Vitro Binding of Egg Albumin

Sera from rabbits immunized with egg albumin, 25 mg/kg, alone or with isoproterenol, 50 micrograms/kg, by footpad injection, were tested for their ability to bind egg albumin *in vitro*. The sera were PCA negative and had PHA titers of 11 and 12 respectively. A 2 ml

volume of serum, either undiluted or diluted 1:10 with 0.85% NaCl, was mixed with an equal volume of egg albumin, 200 micrograms/ml, and allowed to stand at 4^{o} C for 4 or 24 hours.

The back of a rabbit sensitized to egg albumin by the footpad injection of egg albumin 25 mg/kg and propranol 20 mg/kg, was shaved and 0.1 ml volumes of the serum-egg albumin mixtures, egg albumin, saline, and serum were injected into the skin. Evan's Blue dye, 0.5%, 2 ml was injected intravenously at the same time. The reactions were read on the outside of the skin and recorded as described previously.

CHAPTER III

RESULTS

Initial Studies

Initial studies were directed to the determination of the maximum dosage of propranolol tolerated by the rabbit and the best route for administering it. Two animals were injected with 10 mg/kg of propranolol, one intravenously and one subcutaneously. The rabbit receiving the intravenous injection died immediately while the other animal was not visibly affected. Additional rabbits were injected subcutaneously with 15, 20, 25, or 30 mg/kg of propranolol. Within 15 minutes after the injection, rabbits receiving 25 or 30 mg/kg became prostrate, with head drawn back and the legs rigidly extended. Approximately 45 minutes later both animals were able to stand upright and they appeared normal. As the result of this study, 20 mg/kg was selected as the maximum dosage of propranolol.

Two rabbits were immunized with a mixture of propranolol 20 mg/kg and egg albumin 25 mg/kg in the footpads. Because of technical difficulties one of the animals received only one-fourth the intended dosage. A third rabbit was injected with egg albumin, 25 mg/kg, and Freund's complete adjuvant in the footpads. The fourth rabbit received egg albumin, 25 mg/kg, and 0.85% NaCl in the footpads.

Homocytotropic antibody, as demonstrated by the homologous passive cutaneous anaphylaxis (PCA) reaction, was produced only by the rabbits receiving propranolol (Table 1). Both animals had $\log_2$ titers of 8 on the twelfth day after immunization and titers of 4 on the thirtieth day after immunization. No attempt was made to measure the diameters of the areas of bluing in this initial experiment because of the large amount of fur on the skin of the test animals. In subsequent experiments more attention was given to shaving the skin clean before the serum injections were made.

All four of the animals produced antibodies which agglutinated red blood cells sensitized with egg albumin (Table 2). However the rabbits receiving egg albumin with either Freund's complete adjuvant or propranolol had higher antibody titers. The animal which received antigen in saline made only a minimal response.

In the next experiment, animals were immunized in the footpads with egg albumin, 25 mg/kg, in saline, Freund's complete adjuvant, or 20 mg/kg of propranolol. The saline and Freund's complete adjuvant groups consisted of two animals each, and the propranolol group, three animals. The animals were bled at two to three day intervals beginning on the eighth day after immunization. Antibody activity was detected by a qualitative PCA procedure using undiluted serum. Results are shown in Table 3. PCA antibody could be demonstrated consistently only in the serum of the animals receiving propranolol or Freund's complete adjuvant. One animal which received egg albumin in saline had PCA activity on day 8 only, while the other animal in this group showed activity from day 18 to the end of the

TABLE 1

EFFECT OF PROPRANOLOL ON HOMOCYTOTROPIC ANTIBODY FORMATION IN RABBITS

Animal Number	Mixture Injected	Log_2 PCA Antibody Titer[a] Days Post-Immunization					
		0	12	15	18	21	30
1	Propranolol, 5 mg/kg Egg Albumin, 6.2 mg/kg	-1	8	5	7	5	4
2	Propranolol, 20 mg/kg Egg Albumin, 25 mg/kg	-1	8	7	6	6	4
3	Freund's Complete Adjuvant Egg Albumin, 25 mg/kg	-1	-1	-1	-1	-1	-1
4	Saline, Egg Albumin, 25 mg/kg	-1	-1	-1	-1	-1	-1

[a] Log_2 Antibody Titer = $-Log_2$ of highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

TABLE 2

EFFECT OF PROPRANOLOL ON HEMAGGLUTINATING ANTIBODY PRODUCTION IN THE RABBIT

Animal Number	Mixture Injected	Log^2 Antibody Titer[a] Days Post-Immunization 0	12	15	18	21	30
1	Propranolol, 5 mg/kg Egg Albumin, 6.2 mg/kg	-1	9	7.5	7	8	7.5
2	Propranolol, 20 mg/kg Egg Albumin, 25 mg/kg	-1	10.5	10	10	9.5	10
3	Complete Freund's Adjuvant Egg Albumin, 25 mg/kg	-1	9	10	11	12	12
4	Egg Albumin, 25 mg/kg	-1	2	1	1.5	2	1

[a] Log_2 Antibody Titer = $-Log_2$ of highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

TABLE 3

EFFECT OF PROPRANOLOL ON HOMOCYTOTROPIC ANTIBODY FORMATION IN THE RABBIT, QUALITATIVE ASSAY

Animal Number	Adjuvant	PCA, Undiluted Serum[a] Days, Post-Immunization							
		0	8	10	12	15	18	21	31
1	Freund's Complete Adjuvant	0	+	+	+	+	+	+	+
3	"	0	+	+	+	+	+	+	+
4	Propranolol, 20 mg/kg	0	+	+	+	+	+	+	+
5	"	0	+	+	+	+	+	+	+
6	"	0	+	+	+	+	+	+	+
7	None	0	0	0	0	0	+	+	+
8	"	0	+	0	0	0	0	0	0

[a] 0 = no reaction, + = positive reaction

experiment. Sera from all of the animals appeared to be negative for PCA antibody when an attempt was made to titrate their activity. The procedure was repeated and quantitative PCA titers were determined using a new group of recipient animals (Table 4). Titers of from 3 to 6 were seen in the propranolol treated animals. No PCA activity was detected in the serum from one animal receiving complete Freund's adjuvant, and a titer of 4 was observed in the other. Only one of the animals which received egg albumin alone had detectable PCA antibody and this was on days 31 and 35. No explanation can be given for the failure of the first group of test animals to become passively sensitized, or for the apparent loss of activity in some serum samples. All of the animals produced hemagglutinating antibodies, with the highest titers in those which had received Freund's complete adjuvant (Table 5). The maximum $\log_2$ titers were 24 in this group, approximately twice those of the propranolol-treated group, and three to four times those of the group receiving egg albumin alone.

Studies on the Effects of Other Adrenergic Agents on Antibody Formation

Studies were then initiated to examine the effects of other adrenergic agents, including a second beta blocking agent, Sotalol; an alpha blocking agent, phenoxybenzamine; a beta stimulator, isoproterenol; and an alpha stimulator, norepinephrine. The drug dosages used were those reported in the literature as the maximum tolerated dosage or a dosage which produced a physiological response. All the animals received the adrenergic agent mixed with 25 mg/kg of egg albumin via injection of the footpads. The results of this study are

TABLE 4

TITRATION OF HOMOCYTOTROPIC ANTIBODY BY PASSIVE CUTANEOUS ANAPHYLAXIS IN RABBITS

Animal Number	Adjuvant	Log_2 Antibody Titer[a] Days Post-Immunization 0	8	10	12	15	18	21	31	35
1	Freund's Complete Adjuvant	-1	-1	-1	-1	-1	-1	-1	-1	-1
3	"	-1		4		4	4	4	4	4
4	Propranolol, 20 mg/kg	-1		6			6	5	3	4
5	"	-1	6		5		-1	3	5	4
6	"	-1	-1	-1			-1	4	-1	-1
7	None	-1				-1		-1	4	4
8	"	-1	-1	-1						

[a] Log_2 Antibody Titer = $-Log_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

TABLE 5

EFFECTS OF PROPRANOLOL AND COMPLETE FREUND'S ADJUVANT ON HEMAGGLUTINATING ANTIBODY PRODUCTION IN THE RABBIT

Animal Number	Adjuvant	PHA Log_2 Titer[a] Days Post-Immunization 0	8	10	12	15	18	21	31	35
1	Freund's Complete Adjuvant	-1	12	12	12	13	20.5	22	20	21.5
3	"	-1	-1	12.5	13	14	16	24	24	23
4	Propranolol, 20 mg/kg	-1	6	8	7	10	12	12	11.5	12
5	"	-1	8	9.5	9	9	11	16.5	12	12
6	"	-1	5	5	5	7	7.5	8.5	12	12
7	None	-1	5.5	5	6	5	5.5	6	8.5	5
8	"	-1	3.5	5.5	6	6.5	7.5	6.5	2	5.5

[a] Log_2 Antibody Titer = $-Log_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

summarized in Tables 6 and 7. Homocytotropic antibody was produced by all propranolol-treated animals and by four of the five animals receiving phenoxybenzamine. Antibody was detected in both groups on day 7, but the phenoxybenzamine-treated group as a whole responded more slowly than the propranolol-treated group. All of the propranolol-treated animals were producing antibody by day 14, while 4 of 5 phenoxybenzamine-treated animals had responded by day 28.

To test the effect of these drugs on the induction of immunological memory, a second injection of egg albumin, 4 mg/kg, was made into the footpads of each animal on day 28. Homocytotropic antibody activity was detected in a single norepinephrine-treated animal beginning on day 35. Increases in titer were noted in both the propranolol- and phenoxybenzamine-treated groups three weeks after the second antigen injection. Homocytotropic antibody was detected in a single Sotalol-treated animal on day 42 only. None of the isoproterenol-treated animals nor the control animals produced homocytotropic antibody (Table 7). Titers were approximately the same in all groups on day 7, and declined more slowly in the propranolol and phenoxybenzamine groups. Titers increased to approximately the same levels after the secondary immunization.

Selected animals from the previous experiment received a second injection of certain of the adrenergic agents and 10 mg/kg of egg albumin intramuscularly. The purpose of this study was to determine if the agents can influence antibody production quantitatively or qualitatively when they are given together with a tertiary antigenic stimulation. Propranolol and antigen were injected at the same

TABLE 6

EFFECTS OF ADRENERGIC AGENTS ON HOMOCYTOTROPIC ANTIBODY FORMATION

Agent	Number of Animals Responding/Number of Animals in the Group (Mean PCA Log_2 Titer)[a,b] Days Post-Immunization							
	0	7	14	21	28[c]	35	42	49
None	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Propranolol 20 mg/kg	0/5	4/5(3)	5/5(2.4)	4/5(3.5)	4/4(6.5)	2/4(5)	3/4(2)	3/4(8)
Phenoxybenzamine 20 mg/kg	0/5	2/5(1)	3/5(4.7)	3/5(4.7)	4/5(6)	3/5(3)	4/5(3.5)	4/5(5)
Sotalol 20 mg/kg	0/5	0/5	0/5	0/5	0/5	0/5	1/5(0)	0/5
Norepinephrine 120 micrograms/kg	0/4	0/4	0/4	0/4	0/4	1/4(4)	1/4(4)	1/4(4)
Isoproterenol 50 micrograms/kg	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

[a] Log_2 Antibody Titer = $-Log_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

[b] Mean calculated for responders only.

[c] Egg albumin, 4 mg/kg, injected into the footpads.

TABLE 7

EFFECTS OF ADRENERGIC AGENTS ON HEMAGGLUTINATING ANTIBODY FORMATION

Agent	Mean and Range of Log_2 PHA Titer[a] Days Post-Immunization							
	0	7	14	21	28[b]	35	42	49
None	-1	6.5 (3-10.5)	5.2 (4-7)	4 (2-6)	2.6 (2-4)	12.4 (11-14)	11 (9-12)	7.9 (7-8-5)
Propranolol 20 mg/kg	-1	9 (8-10)	9.6 (8-11)	8.9 (8-11.5)	7.5 (6-0)	13.8 (11-15)	11.9 (11.5-12)	10.4 (9.5-11.5)
Phenoxy-benzamine 20 mg/kg	-1	7 (1-10)	7 (5-9)	6.6 (4-9)	5.6 (3-7)	14.4 (14-16)	14.8 (12-19)	12.5 (8.5-16)
Sotalol 20 mg/kg	-1	8 (6-12)	5.6 (4-7)	5.2 (4-7)	3.6 (2-6)	14.8 (12-18)	10.8 (9-14)	8.7 (7-10)
Norepinephrine 120 micrograms/kg	-1	6 (3-9)	5.3 (3-7)	3.9 (2.5-6)	2.8 (2-4)	11 (10-12)	9.9 (7-12)	8 (7-10)
Isoproterenol 50 micrograms/kg	-1	7.9 (7-8)	5.5 (5-6)	4.8 (4-6)	3.3 (2-4)	14 (12-19)	11 (9.5-13)	8.9 (7-10)

[a] Log_2 Antibody Titer = $-Log_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

[b] Egg albumin, 4 mg/kg, injected into the footpads.

site or at separate sites. Isoproterenol was injected either with or without egg albumin. Because the phenoxybenzamine was dissolved in ethyl alcohol, the effect of ethyl alcohol when injected with antigen on antibody production was also evaluated.

Results of this experiment are shown in Table 8. One animal which received propranolol and egg albumin in the same site responded with homocytotropic antibody production. A $\log_2$ antibody titer of six was detected 14 days after immunization. One animal which received egg albumin suspended in ethyl alcohol began producing homocytotropic antibody on day 14 also. Isoproterenol with egg albumin, propranolol and egg albumin at separate sites or egg albumin alone did not initiate homocytotropic antibody production. One animal injected with isoproterenol alone had a 16-fold decrease in PCA antibody activity during the first week after treatment. All animals responded with increases in PHA antibody titers, including an isoproterenol treated animal which received no egg albumin.

The effect of varying the dosage of phenoxybenzamine and propranolol was studied next. Twenty-five mg/kg of egg albumin, along with 40, 4.0 or 0.4 mg/kg of phenoxybenzamine or 20, 2.0 or 0.2 mg/kg of propranolol, were injected into the footpads of groups of rabbits. In the 20 mg/kg propranolol group, 3 of 5 animals were producing homocytotropic antibody by day 10, with a mean titer of 1.6 (Tables 9 and 10). By day 20 all of the animals in this group had responded, with a mean titer of 4.4. The animals receiving 2.0 and 0.2 mg/kg did not produce homocytotropic antibody. In the phenoxybenzamine groups, 1 of 4 animals receiving 4.0 mg/kg and 2 of 4

TABLE 8

EFFECT OF A SECOND INJECTION OF ADRENERGIC AGENT AND ANTIGEN ON ANTIBODY FORMATION IN THE RABBIT

Agent[c]	Weeks After Second Injection	No. of Animals With PCA Antibody/ No. in Group	Mean & Range of PCA Log_2 Titer[a,b]	Mean & Range of PHA Titer[a]
Propranolol and Antigen, Same Site	0	0/4	-1	8(7-10)
	1	0/4	-1	14(12-17)
	2	1/4	6	11.6(10-14)
Isoproterenol and Antigen, Same Site	0	1/3	4	10.8(9.5-12)
	1	1/2	4	12
	2	1/2	4	11
Isoproterenol, No Antigen	0	1/1	6	8.5
	1	1/1	2	12
	2	1/1	2	10
Ethyl Alcohol and Antigen, Same Site	0	1/4	4	7.9(7-8.5)
	1	1/3	2	8.8(6-13)
	2	2/3	2(2)	9.3(6-14)
Propranolol and Antigen, Separate Sites	0	0/4	-1	8.9(7-10)
	1	0/4	-1	14.8(11-18)
	2	0/4	-1	12.3(11-14)
Antigen, Alone	0	2/7	5(4-6)	10.6(7-16)
	1	2/7	5(4-6)	15(12-17)
	2	2/7	5(4-6)	11.4(11-12)

[a] Log_2 Antibody Titers = $-Log_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

[b] Mean and range calculated for responders only.

[c] Agent dosages: 1) antigen-egg albumin, 10 mg/kg, 2) Propranolol-20 mg/kg, 3) Isoproterenol, 75 micrograms/kg, 4) Ethyl alcohol, absolute,-1 ml.

TABLE 9

EFFECT OF VARYING THE DOSAGE OF PROPRANOLOL AND PHENOXYBENZAMINE ON THE IMMUNE RESPONSE IN RABBITS

Agent	Number of Animals Responding[a,b]/Number in Group Days Post-Immunization 0	5	7	10	14	15	20	21
Propranolol								
	a b	a b		a b		a b	a b	
20 mg/kg	0/5 0/5	0/5 5/5		3/5 5/5		4/5 5/5	5/5 5/5	
2.0 mg/kg	0/5 0/5	0/5 5/5		0/5 5/5		0/5 5/5	0/5 5/5	
0.2 mg/kg	0/5 0/5	0/5 2/5		0/5 5/5		0/5 5/5	0/5 5/5	
Phenoxybenzamine								
	a b		a b		a b			a b
40 mg/kg	0/3 0/3		0/3 0/3		0/3 0/3			1/3 2/3
4.0 mg/kg	0/3 0/3		1/4 4/4		3/4 4/4			1/4 4/4
0.4 mg/kg	0/3 0/3		2/4 4/4		4/4 4/4			3/4 4/4

[a] PCA antibody

[b] Hemagglutinating antibody

TABLE 10

EFFECT OF VARYING THE DOSAGE OF PROPRANOLOL AND PHENOXYBENZAMINE ON PASSIVE CUTANEOUS ANAPHYLAXIS ANTIBODY FORMATION IN THE RABBIT

	Mean and Range of Log_2 Antibody Titer[a,b]							
	Days Post-Immunization							
Agent	0	5	7	10	14	15	20	21
Propranolol								
20 mg/kg	-1	-1		1.6(2-4)		2.4(2-4)	4.4(2-6)	
2.0 mg/kg	-1	-1		-1		-1	-1	
0.2 mg/kg	-1	-1		-1		-1	-1	
Phenoxybenzamine								
40 mg/kg	-1		-1		-1			2(2)
4.0 mg/kg	-1		0(0)		4(0-6)			4(4)
0.4 mg/kg	-1		2.5(2-3)		3(0-4)			5.3(4-6)

[a] Log_2 antibody titer = $-Log_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

[b] Number of animals per group: 1) Propranolol-five, 2) Phenoxybenzamine-40 mg/kg, three, 4.0 and 0.4 mg/kg, four each.

animals receiving 0.4 mg/kg were producing homocytotropic antibody by day 7. Mean $\log_2$ titers were 0 and 2.5, respectively. By day 14, 3 of 4 animals receiving 4.0 mg/kg and 4 of 4 receiving 0.4 mg/kg had responded. Mean $\log_2$ titers were 4 and 3, respectively. No antibody was detected in the group receiving 40 mg/kg until day 21, the last day of the experiment, when a single animal had a titer of 2. All animals receiving propranolol were producing PHA antibodies by day 10. All of the phenoxybenzamine treated animals, except the group receiving 40 mg/kg had responded by day 7. Only 2 of 3 animals in the later group were producing hemagglutinating antibodies by day 21, and the maximum mean $\log_2$ antibody titer observed in this group was rather low (Table 11). PHA antibody titers were highest in the 20 mg/kg propranolol group and the 0.4 mg/kg phenoxybenzamine group. The immunosuppressive effect of 40 mg/kg of phenoxybenzamine is quite evident.

Attempt to Define Mechanisms of Phenoxybenzamine and Propranolol Action

To determine if the adjuvant effect of propranolol and phenoxybenzamine was due to a decrease in cAMP levels, dibutyryl cAMP was administered at a dosage of 44 mg/kg into the footpads of a group of rabbits 30 minutes prior to immunization. Control animals not receiving dibutyryl cAMP were injected with 0.25 ml of saline per footpad. The animals were immunized with egg albumin, 25 mg/kg, and either propranolol, 20 mg/kg, or phenoxybenzamine, 0.4 mg/kg. Control animals received egg albumin alone, egg albumin and dibutyryl cAMP, egg albumin and phenoxybenzamine or egg albumin and propranolol.

Two of five rabbits receiving propranolol and dibutyryl cAMP

TABLE 11

EFFECT OF VARYING THE DOSAGE OF PROPRANOLOL AND PHENOXYBENZAMINE ON THE FORMATION OF HEMAGGLUTINATING ANTIBODY IN RABBITS

	Mean and Range of Log_2 Antibody Titers[a,b] of Responding Animals							
	Days Post-Immunization							
Agent	0	5	7	10	14	15	20	21
Propranolol								
20 mg/kg	-1	3.8(2-5)		8(7-9)		7.4(6-9)	7.7(6-9.5)	
2.0 mg/kg	-1	3.6(2-5)		6.4(5-7)		5.3(4-6)	5.4(4-6)	
0.2 mg/kg	-1	1(0-3)		4(3-6)		4.4(2-6)	5.2(4-6)	
Phenoxybenzamine								
40 mg/kg	-1		-1		-1			3.3(1-5)
4.0 mg/kg	-1		5.5(1-8)		7(6-9)			6.1(5-7)
0.4 mg/kg	-1		9.5(8-14)		8.8(8-11)			8.5(6-11)

[a] Log_2 antibody titer = $-Log_2$ of the highest serum dilution showing evidence of antibody activity.[2] For convenience, sera without detectable activity were assigned a titer of -1.

[b] Number of animals per group: 1) Propranolol-five each dosage, 2) Phenoxybenzamine-40 mg/kg, three, 4.0 and 0.4 mg/kg, four each.

were producing homocytotropic antibody on day 7 with a mean titer of 4 (Table 12). The other groups receiving adrenergic agents all contained animals producing homocytotropic antibody by day 14. All animals responded with PHA antibody production by day 7 (Table 13). The mean antibody titer for the phenoxybenzamine treated group was lower than that of the other groups, however the animals which received phenoxybenzamine and dibutyryl cAMP had a mean titer on day 14 approximately equal to that of the group receiving egg albumin alone. Mean antibody titers for the groups receiving propranolol alone, propranolol and dibutyryl cAMP and dibutyryl cAMP alone remained high on day 14, or increased slightly. This experiment was terminated on day 14 because of the deaths of a large number of animals due to unknown causes.

Titration of Skin Test Materials

A rabbit immunized three weeks previously with egg albumin, 25 mg/kg, and propranolol, 20 mg/kg, by footpad injections, received intradermal injections of 0.1 ml volumes of the following test materials: 1) Histamine, 0.0055 to 5,500 micrograms/0.1 ml in tenfold increments, 2) Egg albumin, 0.01 to 10,000 micrograms/0.1 ml in tenfold increments, and 3) Bovine serum albumin, 0.01 to 10,000 micrograms/0.1 ml in tenfold increments. Following the series of injections the animal was given an intravenous injection of 2 ml of Evan's Blue dye. One hour later the areas of bluing were measured through the skin and recorded as described above. The results are given in Table 14. The concentrations selected for further studies were histamine, 0.55 micrograms/0.1 ml, egg albumin, 10 micrograms/0.1 ml, and bovine serum albumin, 10 micrograms/0.1 ml. It was felt that these intermediate

TABLE 12

EFFECTS OF DIBUTYRYL CYCLIC-AMP AND ADRENERGIC AGENTS ON HOMOCYTOTROPIC ANTIBODY FORMATION IN THE RABBIT

Agents[b]	Number of Responding Animals/Number of Surviving Animals, Mean PCA Titer of Responding Animals[a,c]		
	Days Post-Immunization		
	0	7	14
Saline	0/3	0/3	0/2
Propranolol 20 mg/kg	0/3	0/3	2/3, 5(4-6)[c]
Propranolol 20 mg/kg & diB c-AMP 44 mg/kg	0/5	2/5, 4(4)	3/5, 4(2-6)
Phenoxybenzamine 0.4 mg/kg	0/3	0/3	1/1, 2
Phenoxybenzamine 0.4 mg/kg & diB c-AMP 44 mg/kg	0/5	0/5	1/3, 4
diB c-AMP 44 mg/kg	0/5	0/5	0/2

[a] Log_2 Antibody Titer = $-\text{Log}_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

[b] All animals received the indicated drug plus 25 mg/kg egg albumin.

[c] Number in parenthesis indicates range of antibody titers within a group.

TABLE 13

EFFECTS OF DIBUTYRYL CYCLIC-AMP AND ADRENERGIC AGENTS ON HEMAGGLUTINATING ANTIBODY FORMATION IN THE RABBIT

Agent[b]	Number of Responding Animals/Number of Surviving Animals, Mean PHA Titer of Responding Animals[a,c]		
	Days Post-Immunization		
	0	7	14
Saline	0/3	3/3, 8.2(5.5-11)	2/2, 5(3-7)
Propranolol, 20 mg/kg	0/3	3/3, 9(7-12)	3/3, 8.3(7-10)
Propranolol, 20 mg/kg & diB c-AMP, 44 mg/kg	0/5	5/5, 9.8(9-11)	4/4, 9.5(7-11)
Phenoxybenzamine, 0.4 mg/kg	0/3	3/3, 3.2(2-5)	1/1, 1
Phenoxybenzamine, 0.4 mg/kg & diB c-AMP, 44 mg/kg	0/5	5/5, 5.3(2-8.5)	3/3, 5.7(1-9)
diB c-AMP, 44 mg/kg	0/5	5/5, 8.1(4-11)	2/2, 8.5(8-9)

[a] Log_2 Antibody Titer = $-Log_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

[b] All animals received the indicated drug and 25 mg/kg egg albumin.

[c] Number in parenthesis indicates range of antibody titers within a group.

TABLE 14

TITRATION OF EGG ALBUMIN, BOVINE SERUM ALBUMIN AND HISTAMINE BY DIRECT SKIN TEST OF A SENSITIZED RABBIT

	Dosage, microgram[a]	Diameter of Bluing, mm[b]
Histamine	5,500	15
	550	12
	55	12
	5.5	10
	0.55	7
	0.055	5
	0.0055	3
Egg Albumin	10,000	20
	1,000	17
	100	9.5
	10	8
	1	6.5
	0.1	4
	0.01	3
Bovine Serum Albumin	10,000	5
	1,000	3
	100	10
	10	5
	1	0
	0.1	0
	0.01	0

[a] Amount injected in 0.1 ml volume.

[b] Average of two measurements, in millimeters, of the diameter of bluing.

doses would allow detection of both increased and decreased reactivity.

Effects of Propranolol on the Direct Skin Test and the Reaction to Histamine

A group of rabbits which had been immunized as described previously, were tested, by direct skin test, for their immediate hypersensitivity response to egg albumin, 10 micrograms/0.1 ml, bovine serum albumin, 10 micrograms/0.1 ml, and to histamine, 0.55 micrograms/0.1 ml, before and 30 minutes after the injection of propranolol, 20 mg/kg.

The results are given in Table 15. Propranolol treatment in many cases seemed to lessen the response to egg albumin as evidenced by a decrease in diameter of the area of bluing. The response to histamine was also decreased in a number of animals, but to a lesser extent. The response to BSA and saline was not affected. There is no correlation between homocytotropic or hemagglutinating antibody titers and the diameter of the skin reaction. Many animals which did not have detectable antibody had positive skin reactions to egg albumin.

Tests for Blocking Antibody

These procedures were developed to test the possibility some class of antibody might block immediate hypersensitivity by occupying tissue sites competitively. Sera, collected on day 0, 7, 28 and 49 from animals immunized as described previously with adrenergic agents and egg albumin, were injected into the skin of a rabbit sensitized to egg albumin. Thirty minutes later, following the intravenous injection of Evan's Blue dye, 0.1 ml of egg albumin (10 micrograms) was injected into each site. One hour later the rabbit was killed and the

TABLE 15

EFFECT OF PROPRANOLOL ON SKIN TEST REACTIONS IN RABBITS IMMUNIZED WITH EGG ALBUMIN

Animal Number	PCA Titer[a]	PHA Titer[a]	Skin Test Material[b] Diameter of Bluing, Before[c] and After[d] Propranolol							
			EA		BSA		Histamine		Saline	
			c	d	c	d	c	d	c	d
2	-1	6	20	7	2	1	15	18	1	0
3	-1	6	16	11	2	3	16	15	1	0
4	6	8	18	10	2	2	18	15	2	0
5	-1	5	10	18	10	8	25	20	20	20
6	-1	5	20	18	2	2	20	18	2	2
7	2	10	15	11	2	2	16	18	0	0
8	-1	4	14	12	2	2	10	10	0	0
9	-1	6	18	17	1	1	7	5	1	0
10	-1	4	15	8	2	1	18	14	0	0
12	2	8	15	12	0	0	20	18	0	0
13	-1	5	5	5	2	2	24	23	0	0
14	-1	6	23	12	1	1	5	18	0	0
15	6	7	15	11	2	1	10	10	2	0
16	-1	6	2	2	1	2	20	16	2	0
17	6	10	6	5	22	20	9	10	0	0
18	4	11	2	1	20	15	1	10	0	0

[a] Log_2 Titers, where -1 = no reaction; 0 = positive with undiluted serum.

[b] EA = egg albumin, 10 micrograms; BSA = bovine serum albumin, 10 micrograms; Histamine, 0.55 micrograms; saline = 0.85% NaCl. All materials were injected intradermally in 0.1 ml volume.

[c] Average of two measurements, in millimeters, of the diameter of bluing.

[d] Average of two measurements, in millimeters, of the diameter of bluing at a second site, 30 minutes after the footpad injection of propranolol, 20 mg/kg of body weight.

areas of bluing were recorded as described previously.

The results are given in Table 16. There was no evidence that adrenergic drugs stimulate the production of blocking antibodies, at least as measured by this test.

In another experiment undiluted serum was injected into the skin of a sensitized rabbit as described previously. The prepared sites and uninjected sites were challenged at ½, 6, 22, or 41 hours with egg albumin, 10 micrograms/0.1 ml. Increasing the time between serum injection and challenge with egg albumin did not affect the skin test response (Table 17). No inhibition of the skin test response to egg albumin could be shown.

In Vitro Binding of Egg Albumin

Sera from PCA negative rabbits were tested for their ability to bind egg albumin *in vitro*. Serum, undiluted and diluted 1:10 with 0.85% NaCl was mixed with an equal volume of egg albumin, 200 micrograms/ml, and allowed to stand at 4° C for 4 or 24 hours. The serum-egg albumin mixtures, egg albumin, serum, and saline were injected into the skin of a sensitized rabbit as described previously. No differences in the immediate hypersensitivity reactions compared with that produced by egg albumin alone were noted. There was no reaction or very slight response (1-5 mm) to serum alone or saline. The diameters of the areas of bluing in response to egg albumin, and undiluted or diluted serum-egg albumin mixtures were from 10 to 15 mm. Sera from all the bleedings (day 0, 7 and 63) and from both rabbits gave approximately the same results.

TABLE 16

THE EFFECTS OF ADRENERGIC AGENTS ON THE DEVELOPMENT OF BLOCKING ANTIBODY FOR THE IMMEDIATE SKIN TEST REACTION TO EGG ALBUMIN

Agent[a]	Serum Number	Day of Serum Collection — Diameter of Bluing[d] 0 b	0 c	7 b	7 c	28 b	28 c	49 b	49 c
Saline	1	8	7	9	8	6	5	5	6
	2	8	7	9	6	8	9	8	8
Sotalol, 20 mg/kg	1	9	10	9	9	7	9	7	8
	2	9	7	8	9	7	7	5	8
Norepinephrine, 120 micrograms/kg	1	8	6	8	7	8	7	7	8
	2	7	8	8	8	9	9	7	8
Isoproterenol, 50 micrograms/kg	1	8	6	6	8	7	8	7	7
	2	7	6	8	8	6	7	8	8

[a] All the animals were immunized with the indicated drug and egg albumin, 25 mg/kg in the footpads; a second injection of egg albumin was given in the footpads on day 28.

[b] Undiluted serum.

[c] Serum diluted 1:5 in 0.85% NaCl.

[d] Average of two measurements, in millimeters, of the diameter of bluing.

TABLE 17

EFFECT OF PRIOR INTRADERMAL INJECTION OF HYPERIMMUNE, PCA NEGATIVE SERUM ON THE SKIN TEST REACTION IN SENSITIZED RABBITS

Period of Fixation Prior to Challenge of Injected Site	Diameter of Bluing in Millimeters	
Hours	Serum Injected Site	Normal Site
½	11	10
6	8	9
22	8.5	10
41	10	9

Physicochemical Properties of Antibodies from Phenoxybenzamine and Propranolol Treated Animals

The heat stability, mercaptoethanol sensitivity and latent period required for skin fixation of the homocytotropic antibody were studied. Serum pools from early and late bleedings were prepared for both propranolol and phenoxybenzamine treated groups. Heating at 56° C decreased PCA activity but did not markedly lower hemagglutination titers (Table 18). Complete inactivation of the PCA activity of sera collected on day 7 was accomplished in two hours while sera collected on day 49 still gave a positive reaction when injected undiluted. The magnitude of the decrease in titer was about the same for both early (day 7) and late (day 49) sera.

Reduction with 2-mercaptoethanol followed by alkylation with 0.02 M iodoacetamide completely inactivated the homocytotropic antibody, lowering titers from 1 or 2 to -1 (Table 19). Passive hemagglutination titers of sera collected on day 7 were similarly reduced from 7 to 1 or 2. Day 49 PHA titers were not lowered by this treatment.

PCA reactions were obtained when antigen was given as early as 24 hours after the injection of serum (Table 20). These reactions were faint and usually confined to undiluted serum. At 48 and 72 hours, reactions were obtained with more dilute sera. No difference could be found in the sera from propranolol treated and phenoxybenzamine treated animals.

TABLE 18

EFFECTS OF HEAT ON ANTIBODY TITER IN SERUM FROM RABBITS RECEIVING PROPRANOLOL OR PHENOXYBENZAMINE AT THE TIME OF IMMUNIZATION

Group	Day of Serum Collection	Hours Heated	PCA Titer[a]	PHA Titer[a]
Propranolol	7	0	1	9
		1	0	9
		2	-1	9
		4	-1	9
	49	0	2	13
		1	1	13
		2	1	12
		4	0	12
Phenoxybenzamine	7	0	0	13
		1	0	13
		2	-1	12
		4	-1	12
	49	0	2	20
		1	1	19
		2	1	17
		4	0	16

[a] Log_2 Antibody Titer = $-\text{Log}_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

TABLE 19

EFFECT OF REDUCTION AND ALKYLATION ON ANTIBODY TITERS IN RABBITS RECEIVING ADRENERGIC AGENTS AT TIME OF IMMUNIZATION

Mixture Injected	Day of Serum Collection	Log_2 PCA Titer[a]		Log_2 PHA Titer[a]	
		Before Red. & Alk.	After Red. & Alk.[b]	Before Red. & Alk.	After Red. & Alk.[b]
Propranolol 20 mg/kg & egg albumin 25 mg/kg	7	2	-1	7	1
	49	1	-1	12	12.5
Phenoxybenzamine 0.4 mg/kg & egg albumin 25 mg/kg	7	2	-1	7	2
	49	2	-1	13.5	11.5

[a] Log_2 Antibody Titer = $-Log_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

[b] Red. & Alk. = Reduction with 0.1 M 2-mercaptoethanol for 3 hours followed by alkylation with 0.02 M iodoacetamide for 4 hours.

TABLE 20

LATENT PERIOD REQUIRED FOR SKIN FIXATION OF HOMOCYTOTROPIC ANTIBODY IN THE RABBIT

Mixture Injected	Day of Serum Collection	Log_2 PCA Antibody Titer[a] Hours Post Serum Injection		
		24	48	72
Propranolol, 20 mg/kg & Egg Albumin, 25 mg/kg	7	0	1	3
	49	1	2	2
Phenoxybenzamine, 20 mg/kg & Egg Albumin, 25 mg/kg	7	-1	1	2
	49	0	1	2

[a] Log_2 Antibody Titer = $-Log_2$ of the highest serum dilution showing evidence of antibody activity. For convenience, sera without detectable activity were assigned a titer of -1.

CHAPTER IV

DISCUSSION

Adrenergic drugs have been reported previously to both enhance and suppress the immune response (53-61). The present study confirms these reports and presents new data on the effects of adrenergic blockade on homocytotropic antibody formation.

An increase in cAMP levels either as the result of isoproterenol stimulation or by endogenous cAMP dynamics has been reported to both suppress and enhance antibody formation (55, 58, 59). Similar findings have been noted for the alpha adrenergic stimulator, norepinephrine, which depresses cAMP levels (56, 57). In the present study, neither of these drugs appeared to influence the production of PHA antibodies for egg albumin. This could have been the result of a suboptimal drug dosage or period of drug exposure. In the study of Reed *et al.* (55), which revealed an immunosuppressive effect for isoproterenol and epinephrine, the drugs were given at 8 and 6 hour intervals for 10 days.

The immunological adjuvanticity of propranolol when administered at dosages of 5 to 20 mg/kg of body weight at the time of antigen exposure is confirmed in the present study. Titers of PHA antibody achieved with propranolol were exceeded only by those

obtained with complete Freund's adjuvant (CFA). Antibody titers declined much more slowly in the propranolol- and CFA-treated groups. The response of propranolol-treated animals to a second injection of antigen was not different from that of animals treated with other adrenergic agents.

For the first time, propranolol has been demonstrated to be an adjuvant for homocytotropic antibody formation. The reason for this rests probably with the timing and duration of the treatment with propranolol. In previous studies, propranolol was given for a period of 10 days after immunization. Failure to enhance homocytotropic antibody formation by long term beta adrenergic blockade seems inconsistent with the theory of Szentivanyi (20). It is possible, however, that beta blockade in the atopic individual involves only a specific cell type participating in the immune response. If so, a single injection of propranolol, as used in the present study, might reproduce this selective block. Such a selective blockade might not be invoked by the administration of propranolol for a longer period of time. Exposure to propranolol at the time of antigen injection apparently influences cells involved in the initiation of the immune response without being inhibitory for later cell functions.

The immune response is thought to involve interactions among three types of cells (120). These include macrophages, which may concentrate antigen or present antigen to lymphocytes for immune recognition, and lymphocytes. Lymphocytes are thought to be of at least two types, antigen-reactive cells which process antigen in some fashion and antibody forming cells. Beta adrenergic blockade at the time

of immunization could cause the proliferation of any one, or all three of these cell types. Beta adrenergic blockade might alter the function of these cell types. These events are most likely related to cellular cAMP levels.

Shepard (121) has reported that transformed cells have lower cAMP levels than normal cells and that agents which decrease cellular cAMP levels stimulate growth. Such findings suggest that biochemical events which involve high concentrations of cAMP promote cellular differentiation and that the lowering of cAMP levels allows events associated with cell proliferation to proceed.

Hadden et al. (122) found that the phytohemagglutinin and concanavalin A initiated proliferation of peripheral blood lymphocytes is accompanied by a ten to fiftyfold increase in guanosine 3': 5'-cyclic monophosphate (cGMP) concentration in the cells. No changes in cAMP levels were noted. Strom et al. (123) noted an increase in cGMP levels following the addition of cholinergic agents to rat lymphocytes in vitro. The interrelationship of cAMP and cGMP is not known, but Franks and MacManus (124) reported that the hydrolysis of cAMP by phosphodiesterase is enhanced by cGMP in vitro.

Whatever the mechanism by which beta adrenergic blockade enhances immunoglobulin production, the cells involved in homocytotropic antibody formation appear to be most affected. Reed (125) has proposed that adrenergic blockade in atopic individuals involves the cells which produce IgE.

The beta adrenergic blocking drug Sotalol did not exert an adjuvant effect on the primary immune response. However, homocytotropic

antibody was detected in a single rabbit on day 42 after a second injection of egg albumin (Table 6). Lack of adjuvanticity may be related to the dosage administered or to the fact that Sotalol is a more selective blocking agent. Sotalol appears to block the beta-1 subtype of beta receptors. This receptor mediates inotropic, chronotropic, cardiac and lipolytic effects. Propranolol blocks both beta-1 and beta-2 mediated effects, and it is possible that a beta-2 receptor is more involved with homocytotropic antibody production. It has been postulated that the beta-2 receptor responses are impaired in atopic disease (125).

A single norepinephrine treated animal also began producing homocytotropic antibody after a second antigen injection (Table 6). Since norepinephrine also lowers cAMP levels, but by a different mechanism than propranolol, it may have influenced an early event in the immune response, leading to enhancement of homocytotropic antibody formation on second exposure to antigen.

The most interesting and puzzling finding in the present research is that phenoxybenzamine, an alpha adrenergic blocking agent, is also an adjuvant for both PHA and homocytotropic antibody formation. Pieroni and Levine (61) had previously reported that a dosage of approximately 40 mg/kg was immunosuppressive in mice. However, dosages of 3 mg/kg (53) and 0.2 to 0.5 mg/kg (62) (the human therapeutic equivalent) were reported not to affect the humoral immune response in the rabbit. In the present study, 0.4 mg/kg of phenoxybenzamine was found to be a better adjuvant than 20 mg/kg of propranolol for both homocytotropic and PHA antibody formation. As the dosage of phenoxybenzamine was

increased, an effect was noted which was more one of delay than suppression. Even at a dosage of 40 mg/kg, when none of the animals had a detectable antibody titer on day 14, 1 of 3 had a homocytotropic antibody titer and 2 of 3 had PHA antibody titers on day 21 (Table 10).

Phenoxybenzamine does not block beta adrenergic receptors. The fact that it enhances homocytotropic antibody formation without causing a beta blockade suggests either that it lowers cAMP levels through another mechanism or that cAMP is not the compound which is mediating the effect. Both alpha and beta blockade should tend to increase ATP levels. Alpha blockade is related to ATPase inhibition and beta blockade interferes with the conversion of ATP to cAMP. How modification of ATP utilization might increase homocytotropic antibody formation is not known. A second compound which may be important is cGMP. It is also possible that homocytotropic antibody formation could be regulated by two or more separate compounds, acting either through the same of through different mechanisms. The studies of Hadden et al. (122) correlated the mitogenic effects of PHA and concanavalin A with an increase in cGMP levels, accompanied by no change in cAMP levels. The results suggested that an increase in cGMP may represent a signal that induces cell division, while an increase in cAMP inhibits cell division. The important thing may not be the actual concentrations of the two compounds but their relative proportions. This balance could be upset by a beta block which would lower cAMP or by cholenergic stimulation which increases cGMP levels (123). Phenoxybenzamine has been reported to have certain anticholinergic

effects (126). Further studies will be needed to clarify the roles of both cholinergic and adrenergic receptors in the immune response.

Tada et al. (127-131) have postulated that two mechanisms are involved in the control of homocytotropic antibody formation in rats, namely (a) the presence of IgG antibodies at the time of antigenic stimulation or after antigenic stimulation inhibits homocytotropic antibody formation, and (b) thymus-dependent cells have a dual role in the formation of homocytotropic antibody, i.e. they are required during the early events and are suppressive during later events in the differentiation and function of homocytotropic antibody forming cells.

The observed effects of alpha and beta blocking agents fit nicely into these postulates, assuming that T and B cells have different adrenergic requirements. For instance, beta adrenergic block might enhance homocytotropic antibody formation directly by stimulating the activity of B cells, while alpha adrenergic blockade might accomplish the same thing indirectly by enhancing the early, or inhibiting the late activities of T cells which influence homocytotropic antibody formation. Other possibilities exist with these hypothetical mechanisms, but further experimentation would be required to confirm or deny them.

In the present studies an attempt was made to inhibit the adjuvant effect of beta and alpha adrenergic blockade by the administration of dibutyryl cAMP prior to injection of the adrenergic agent and antigen. Dibutyryl cAMP appeared to enhance the adjuvanticity of both propranolol and phenoxybenzamine for homocytotropic antibody

formation. This could be the result of an increase in phosphodiesterase activity in response to the dibutyryl cAMP which, when followed by beta blockade, would lower cAMP levels even further. How this would also increase the effects of alpha blockade is not known.

The effects of a second injection of adrenergic agent and egg albumin on PHA and homocytotropic antibody formation were also studied. Isoproterenol and antigen did not lower homocytotropic antibody titers. Isoproterenol alone had an adjuvant effect on PHA antibody formation while lowering the homocytotropic antibody titer. Propranolol and egg albumin administered together at the same site initiated homocytotropic antibody formation, while the two compounds injected at separate sites did not (Table 8). This suggests that local effects, possibly relating to propranolol concentration, are important in establishing adjuvanticity. The effect of alcohol on homocytotropic antibody formation is interesting since it seemed to lower the antibody titer in one animal and initiate antibody production in another animal (Table 8). This study is difficult to interpret because of the small number of animals in certain groups and the differences in adrenergic treatment during primary immunization.

Propranolol did not enhance the immediate skin test reaction to egg albumin or histamine (Table 15). The <u>in vitro</u> antigen-induced histamine release from sensitized human leukocytes has been shown to be inhibited by compounds which increase cellular cAMP levels (132). One of these inhibitory compounds is histamine. It can be postulated that the histamine liberated by the mast cells in response to the first injection of egg albumin was able to inhibit, to a certain

extent, the histamine release by other mast cells. The beta blockade produced by propranolol was not sufficient to overcome this inhibition and result in a larger skin reaction. The response to histamine should remain the same since there is no information that the actions of this drug are mediated through the adrenergic receptors. No correlation was found between skin test response and homocytotropic antibody titers (Table 15). Most of the animals gave a positive response to egg albumin, even though their serum did not have PCA antibody. This suggests that homocytotropic antibody normally attaches to mast cells as rapidly as it is formed and that only the excess is found in the serum.

No evidence was found for a "blocking" antibody which could bind to egg albumin either *in vitro* or *in vivo* and prevent the immediate skin test response (Tables 16, 17). This is not unexpected since these antibodies are usually found only after a considerable number of antigen injections.

The physiochemical properties of the homocytotropic antibody did not differ remarkably from those reported previously for this antibody in rabbits (117). The antibodies from both propranolol- and phenoxybenzamine-treated animals appeared to be the same. The sequence of immunoglobulin formation was normal, as evidenced by a lowering of PHA antibody titers following mercaptoethanol treatment of serum collected early, but not late in the immunization period. This suggested that the initial response was primarily antibody of the immunoglobulin class IgM, which is mercaptoethanol-sensitive. In the secondary response, the antibody was of the immunoglobulin class IgG, which is mercaptoethanol-resistant.

CHAPTER V

SUMMARY

Propranolol, a beta adrenergic blocking agent, when administered at dosages of 5 to 20 mg/kg of body weight, enhanced both homocytotropic (PCA) and hemagglutinating (PHA) antibody formation in the primary immune response of rabbits to egg albumin. No significant effect on the secondary immune response could be demonstrated. Phenoxybenzamine, an alpha adrenergic blocking agent, at dosages of 0.4 to 40 mg/kg, also had an adjuvant effect. A marked delay in the immune response was noted with high dosages of phenoxybenzamine. The administration of dibutyryl cAMP prior to the injection of propranolol or phenoxybenzamine enhanced the adjuvanticity of these compounds. Sotalol, a selective beta-1 adrenergic receptor blocking agent, and Arterenol, an alpha adrenergic stimulant, did not affect the primary immune response. However, following a second injection of antigen, homocytotropic antibody was detected in a single animal in each group. Isoproterenol, a beta adrenergic stimulant, had no adjuvant effect.

Treatment with propranolol 30 minutes prior to skin testing appeared to lessen the immediate hypersensitivity response of sensitized rabbits to egg albumin and histamine. A positive skin test to egg albumin was demonstrated by many animals even though their serum

did not contain homocytotropic antibody demonstrable by the homologous PCA reaction.

The injection of hyperimmune, PCA-negative serum into the skin of sensitized animals prior to skin testing with egg albumin did not lessen the immediate hypersensitivity response. The incubation of antigen with hyperimmune serum prior to injection did not affect the skin test response to the antigen.

Heating sera at 56° C for periods of up to 4 hours reduced or abolished PCA activity but did not lower PHA antibody titers. Reduction with 2-mercaptoethanol and alkylation with iodacetamide abolished PCA antibody activity and lowered early (day 7) PHA antibody titers. The PHA antibody titers of sera collected on day 49 were not affected by this treatment.

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