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The Production of IgE and IgG1 Antibodies in Guinea-pigs Immunized with Antigen and Bacterial Lipopolysaccharides

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Summary. The adjuvant action of lipopolysaccharide (LPS) on the IgE and IgG1 antibody production by guinea-pigs was studied. It was observed that LPS induces an early preferential production of IgE antibody. The optimal dose of LPS to cause this effect was of the order of 20 μg/kg. Low amounts of LPS as well as low amounts of antigen favoured the production of IgE antibody whereas high amounts of LPS or antigen favoured the production of IgG1. Later, in the course of immunization, the IgE antibody content decreased and the IgG1 antibody content increased. Booster injection of antigen resulted in a considerable and simultaneous increase in both IgE and IgG1. The preferential production of IgE was consistently obtained when LPS and antigen were injected simultaneously. Injection of LPS 24 hours after antigen resulted in a higher production of IgE and IgG1 but in the loss of the preferential production of IgE. Guinea-pig IgE antibody was able to persist in homologous skin for at least 46 days.

INTRODUCTION

The fact that many Gram-negative bacteria exert an adjuvant effect is well known. The adjuvant property of these micro-organisms is in great part due to their content in lipopolysaccharide (LPS). The adjuvant effect of the purified LPS has been observed in rabbits (Johnson, Gaines and Landy, 1956; Kind and Johnson, 1959), mice (Merrit and Johnson, 1963) and guinea-pigs (Farthing and Holt, 1962). In all these papers the adjuvant effect has been studied by determining the total antibody level without discriminating the antibody classes. In the present paper we report the adjuvant effect of bacterial lipopolysaccharide on the IgG1 and IgE antibodies in guinea-pigs. It is shown that LPS induces a preferential early production of IgE antibody.

MATERIAL AND METHODS

Animals

Outbred guinea-pigs of either sex weighing between 250 and 350g reared at the Instituto Butantan animal farm were used throughout.

Antigen

Five times crystallized ovalbumin (Ov) purchased from Pentex Inc. (Kankakee, Ill., U.S.A.) was used as antigen.

Lipopolysaccharide

LPS from *Bordetella pertussis* and *Salmonella typhosa* was prepared by the method of Westphal and Luderitz (1954) as described by Kabat and Mayer (1961). In summary, dried micro-organisms were suspended in 250 ml distilled water at 68°, 250 ml of 90 per cent phenol added and the mixture kept at this temperature with constant stirring for 20 minutes. The mixture was cooled to 5–10°, centrifuged and the upper aqueous phase decanted. The phenol phase was again treated with distilled water at 68°, cooled and centrifuged and the aqueous phase separated. This treatment was repeated once more and all the water extracts combined and dialized in the cold room against large volumes of distilled water until free of phenol. The dialized extract was filtered and concentrated by lyophilization to a small volume. The concentrated extract was then precipitated by addition of 6 volumes of ethanol and a trace of sodium acetate, the precipitate dissolved in distilled water, and the LPS purified by repeated centrifugations at 30,000 rev/min until free of material absorbing at 2,600 μm. The physiological activity of this material was assayed by the Schwartzmann reaction and by its toxicity in adrenalectomized mice.

Lipopolysaccharide B from *S. typhosa* 0901 and from *Escherichia coli* 2023 obtained from Difco Laboratories were also used.

Technique for obtaining serum

Blood was obtained by cardiac puncture or by puncture of the ophthalmic plexus and was allowed to clot in an ice bath. The serum was separated by centrifugation in a refrigerated centrifuge. Serum samples were used as soon as possible or when necessary were kept at $-20°$.

Passive cutaneous anaphylaxia (PCA)

Ovary's technique for PCA (1964) was employed for detecting and estimating antibodies. The dorsal region of the animals was previously shaved with an electrical hair clipper, care being taken to avoid irritation of the skin. Several intradermal injections of antiserum or antiserum dilutions were made on each side of the dorsal skin with a hypodermic needle. After a short latent period (18 hours) or a long latent period (72 hours or 10 days) the animals were injected intravenously with 1 ml of 0·25 per cent solution of Evans' blue in saline (0·15 M NaCl) containing 1 mg antigen. Twenty to 30 minutes after antigen injection, the animals were killed, the skin inverted and the lesion diameter measured on the inner surface of the skin with a transparent ruler. Antibody was estimated by determining the highest dilution of antiserum which induced a PCA reaction. PCA titres are stated as the reciprocal of the highest serum dilution giving a skin reaction. A minimum of three guinea-pigs was used for each determination.

Criteria used for characterization and estimation of IgE and IgG1

Guinea-pig IgE antibody can be distinguished from other homocytotropic antibodies of this species by its thermolability, inactivation by sulphydryl reagents and its ability to remain in the passively sensitized skin for a long period of time (Catty, 1969; Mota and Perini, 1970; Perini and Mota, 1972). In this paper two criteria are used to confirm the presence of IgE antibody in a serum: the loss of its ability to induce PCA reactions after heating (56° for 4 hours) and the long persistency in the passively sensitized skin (10 days). Usually the total loss of the heated serum to produce a PCA reaction after 72 hours was considered sufficient proof for the presence of IgE antibody in the serum. However, when

the loss of PCA activity by heating was only partial, the PCA reactions were repeated using a latent period of 10 days. Only antisera whose long latency PCA activity was completely destroyed by heating and was able to persist in the passively sensitized skin for at least 10 days were considered to contain IgE antibody. To detect IgG1 type antibody the antisera were heated for 4 hours at 56° to destroy PCA activity due to IgE and the heated serum injected for PCA using a short latent period. To estimate the IgG1 antibody content, the antisera showing heat resistant PCA activity were pooled and the PCA titre of the heated pool determined using a short latent period. To estimate the IgE antibody content, the antisera showing heat labile PCA activity were pooled and the PCA titre of the pool determined using a long latent period.

RESULTS

PREFERENTIAL PRODUCTION OF IgE BY LPS

Guinea-pigs were injected intraperitoneally with 5 μg LPS from different sources plus 10 μg Ov. This amount of antigen was chosen because it elicited no detectable serum antibody in control animals. Ten days later the animals were bled and the unheated and heated individual sera diluted 1:5 with saline and assayed for the presence of IgE and IgG1 antibodies as already described. The results of these experiments summarized in Table 1 show that bacterial LPS induces a preferential early production of IgE antibody when used as an adjuvant. The same results suggest that LPS obtained from *S. typhosa* is more efficient in inducing the production of IgE than LPS obtained from *E. coli* or *B. tussis*.

EFFECT OF DIFFERENT DOSES OF LPS ON IgE PRODUCTION

To find out the optimum dose of LPS to induce IgE antibody, groups of guinea-pigs were injected intraperitoneally with a constant amount of antigen and different doses of LPS, bled 10 days later and the IgE and IgG1 content of their sera estimated. As can be seen in Table 2 low doses of LPS tended to induce a preferential production of IgE antibody whereas high doses favoured the production of IgG1 antibody.

EFFECT OF DIFFERENT AMOUNTS OF ANTIGEN

To find out the effect of different amounts of antigen on the induction of IgE antibody guinea-pigs were injected intraperitoneally with 20 μg/kg LPS and different amounts of Ov and bled 10 days later. The results of these experiments shown in Table 3 indicate that whereas low amounts of antigen favour the production of IgE antibody high amounts favour the production of IgG1 antibody. Indeed, when a high dose of Ov (1000 μg) was used the percentage of antisera showing IgE was of the same order as that observed in control animals injected only with the same amount of antigen.

EFFECT OF SEPARATION IN TIME BETWEEN THE INJECTIONS OF ANTIGEN AND LPS

In order to investigate how separate injections of antigen and LPS would affect IgE and IgG1 antibody production groups of guinea-pigs were injected either first with LPS and second with antigen or first with antigen and second with LPS. Ten days later the

TABLE 1

THE EFFECT OF LPS ON THE PRODUCTION OF IgE AND IgG1 IN GUINEA-PIGS. THE ANIMALS WERE INJECTED INTRAPERITONEALLY WITH OVALBUMIN (10 μg) PLUS LPS (5 μg) OBTAINED FROM *S. typhosa*, *B. pertussis* OR *E. coli*, BLED 10 DAYS LATER AND THEIR SERA TESTED FOR PCA ACTIVITY DUE TO IgG1 AND IgE

	LPS from *S. typhosa* PCA activity			LPS from *B. pertussis* PCA activity			LPS from *E. coli* PCA activity	
Anti-sera	IgG1*	IgE†	Anti-sera	IgG1*	IgE†	Anti-sera	IgG1*	IgE†
1	0	+	1	+	+	1	0	0
2	0	+	2	0	+	2	0	+
3	0	+	3	0	0	3	0	0
4	0	0	4	0	0	4	0	0
5	0	0	5	0	0	5	0	0
6	0 7%	0 46%	6	0 9%	0 36%	6	0 8%	0 25%
7	+	+	7	0	+	7	0	0
8	0	0	8	0	0	8	0	0
9	0	0	9	0	0	9	+	+
10	0	+	10	0	0	10	0	+
11	0	0	11	0	+	11	0	0
12	0	0	–	–	–	12	0	0
13	0	+	–	–	–	–	–	–
PCA titre of pools	5	15		5	15		5	5

* PCA activity not destroyed by heat.
† PCA activity completely destroyed by heat.

animals were bled and their sera content of IgE and IgG1 determined and compared with that of animals injected simultaneously with antigen and LPS. As can be seen in Fig. 1 when LPS was injected either 24 hours before antigen or 48 hours after antigen no IgE or IgG1 could be detected in serum. However, injection of LPS 24 hours after antigen resulted in a considerable increase in both IgE and IgG1 antibodies. This increase was revealed by a higher percentage of animals showing IgE and IgG1 in their antisera and by a higher PCA titre for both antibodies. However, in this situation there was no preferential production of IgE.

TIME-COURSE OF IgE IN SERUM

To follow the persistence of IgE antibody in serum of guinea-pigs immunized with

TABLE 2

THE EFFECT OF DIFFERENT DOSES OF LPS ON THE PRODUCTION OF IgE AND IgG1 ANTIBODIES BY GUINEA-PIGS. THE ANIMALS WERE INJECTED INTRAPERITONEALLY WITH 10 μg Ov AND DIFFERENT DOSES OF LPS, BLED 10 DAYS LATER AND THE PERCENTAGE OF ANIMALS SHOWING IgE AND IgG1 IN THEIR SERA DETERMINED

LPS μg/kg	Number of sera tested	Per cent	
		IgG1	IgE
10	20	0	0
20	12	7	50
40	12	1	36
60	12	1	30
120	20	15	0

TABLE 3

EFFECT OF DIFFERENT DOSES OF ANTIGEN ON THE PRODUCTION OF IgE AND IgG1 ANTIBODY BY GUINEA-PIGS. THE ANIMALS WERE INJECTED INTRAPERITONEALLY WITH DIFFERENT AMOUNTS OF ANTIGEN OR ANTIGEN PLUS A CONSTANT DOSE OF LPS, BLED 10 DAYS LATER AND THE PERCENTAGE OF ANIMALS SHOWING IgE AND IgG1 DETERMINED

Ovalbumin μg	LPS μg/kg	Number of sera tested	Per cent	
			IgE	IgG1
1	20	20	0	0
10	20	20	50(15)	10(5)
10	0	20	0	0
1000	20	20	16(5)	90(160)
1000	0	20	17(5)	50(40)

Figures in parentheses represent PCA titre of pools of positive sera.

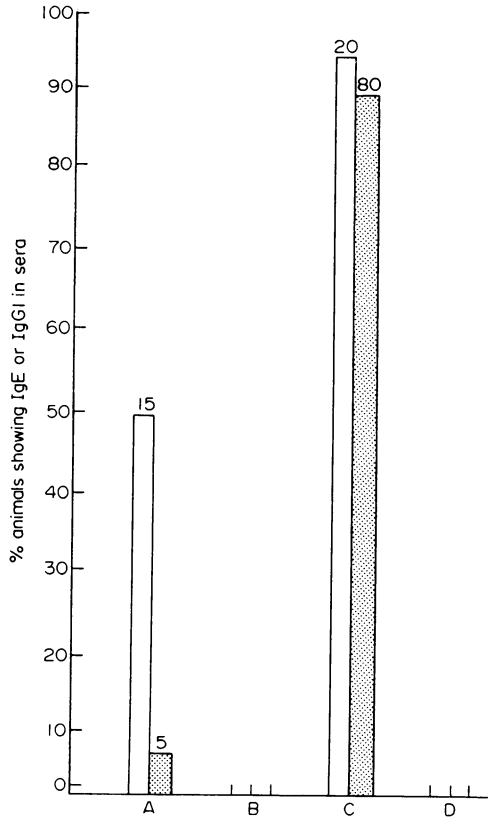

FIG. 1. Effect of separation in time of antigen and LPS injection on the production of IgE (open column) and IgG1 (stipple column) antibody in guinea-pigs. The animals were injected intraperitoneally with 10 μg Ov and 20 μg/kg LPS and bled 10 days later. (A) Production of IgE and IgG1 after simultaneous injection of antigen and LPS. Production of IgE and IgG1 in animals injected first with: (B) LPS and 24 hours later with antigen; (C) antigen and 24 hours later with LPS; (D) antigen and 48 hours later with LPS. Figures over columns represent PCA titre of pools of positive sera.

antigen plus LPS, animals were injected intraperitoneally with 10 μg Ov plus 20 μg/kg LPS and were bled after 10, 30, 60 and 90 days. For each bleeding time the percentage of antisera showing IgE and IgG1 as well as the antibody content of the same were determined. As indicated in Fig. 2, up to 10 days after immunization both the percentage of animals showing IgE antibody in their sera and the IgE content of the same were higher than those of animals showing IgG1 antibody; from then on, though the content of IgE antibody still increased up to the 30 days, there was no more preferential production of IgE antibody since the percentage and the content of IgG1 antibody became higher than those of IgE antibody. After 90 days the level of IgE antibody became very low whereas the level of IgG1 was still high.

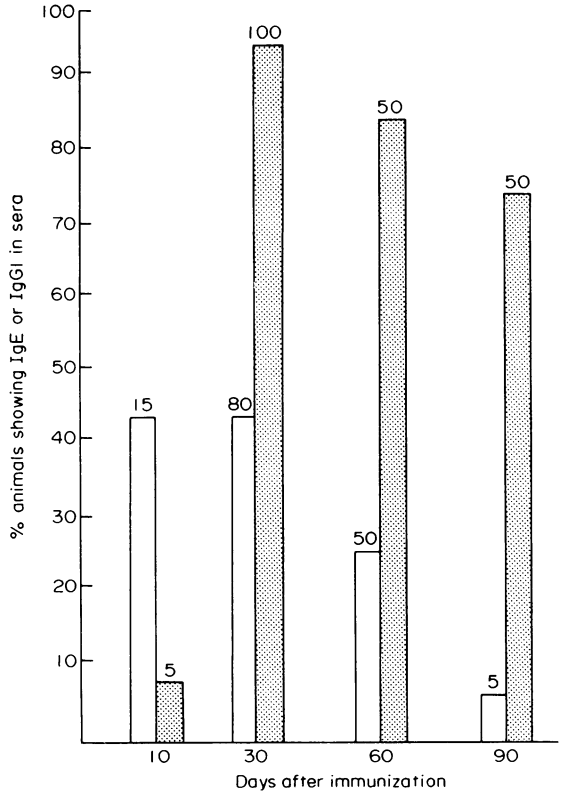

FIG. 2. Time-course of IgE and IgG1 in guinea-pigs sensitized with antigen plus LPS. Presence of IgE (open column) and IgG1 (stipple column) in antisera collected at different times after immunization. The animals were injected intraperitoneally with 10 μg Ov and 20 μg/kg LPS. Figures over columns represent PCA titre of pools of positive sera.

PERSISTENCE OF IgE ANTIBODY IN HOMOLOGOUS SKIN

In order to know how long after passive sensitization would guinea-pig IgE antibody remain in homologous skin, guinea-pigs were injected with guinea-pig antisera containing IgE antibody and challenged at different periods of time after sensitization. Thus, animals were sensitized at days 0, 15, 25 and 36 and challenged 10 days after the last sensitizing

injection. In this way PCA reactions with latent periods of 10, 21, 31 and 46 days could be elicited in the same recipient animal. The results of these experiments summarized in Table 4 show that guinea-pig IgE antibody can remain in the passively sensitized skin for at least 46 days.

TABLE 4

SKIN PERSISTENCE OF GUINEA-PIG IgE ANTIBODY. THE ANIMALS WERE SENSITIZED AT DAYS 0, 15, 25 AND 36 WITH IgE CONTAINING ANTISERUM AND CHALLENGED 10 DAYS AFTER THE LAST SENSITIZING INJECTION

Antisera dilutions	Days after sensitization PCA reactions (mm)			
	10	21	31	46
Und	ND	20×20	20×16	15×15
25	12×12	13×11	10×10	0
50	8×8	10×8	±	0

Und = undiluted serum. ND = not done.

EFFECT OF A SECONDARY DOSE OF ANTIGEN ON IgE AND IgG1 ANTIBODY PRODUCTION

To investigate the behaviour of IgE antibody after a second antigenic stimulation guinea-pigs were injected intraperitoneally with 10 μg Ov plus 20 μg/kg LPS and 90 days later when their IgE sera content was very low they were injected once more with 10 μg Ov intraperitoneally and bled 8 days later. The results of these experiments showed that after a secondary stimulus both the IgE and IgG1 antibody content of the antisera increased more than seventy times over their previous level.

DISCUSSION

It is now well established that guinea-pigs produce homocytotropic antibodies of the IgG and IgE classes (Ovary, Benacerraf and Bloch, 1963; Catty, 1969; Mota and Perini, 1970; Parish, 1970; Dobson, Morseth and Soulsby, 1971; Levine, Chang and Vaz, 1971; Perini and Mota, 1972). The IgE antibody can be distinguished from IgG1 antibodies by being heat labile and able to remain in the passively sensitized skin for many days. Previous work has suggested that antigen and adjuvant are important in preferentially inducing the production of each of these antibodies (Perini and Mota, 1972). Our present findings showing that the use of LPS as an adjuvant results in a preferential production of IgE antibody reinforces this conclusion. The IgE antibody induced by LPS has all the characteristics of mammalian reagins, being heat labile and able to remain in the passively sensitized homologous skin for at least 46 days, being routinely assayed after a latent period of 10 days.

Our results show that the preferential production of IgE antibody is limited to the early phase of the primary antibody response and that later in the course of the immune response the IgE antibody decreases whereas IgG1 antibody increases. A similar relationship in the time-course of IgE and IgG1 antibody production has previously been observed in mice (Mota, Wong, Sadun and Gore, 1969). These findings are in agreement with previous observations showing that the production of IgE antibody is a transient event in the early phase of the primary antibody response in many other species (Mota, 1963, 1964, 1967; Mota and Peixoto, 1966; Zvaifler and Becker, 1966) and suggest that IgG may

exert a feedback regulation on the synthesis of IgE antibody in the guinea-pig. Indeed, regulation of IgE antibody synthesis by IgG has already been described in rats by Tada and Okumura (1970).

The preferential production of IgE antibody induced by LPS clearly depended on the quantitative relationship between antigen and this substance. Thus, when guinea-pigs were injected with 10 μg antigen plus 40 μg or 60 μg/kg LPS and were bled 10 days later reactive animals responded by producing almost only IgE antibody whereas animals injected with a higher dose of LPS (120 μg/kg) responded by producing almost exclusively IgG1 antibody. It is also clear from these experiments that low levels of antigen favour the production of IgE antibody whereas high levels of antigen favour the production of IgG1 antibody. The enhancing effect of low doses of antigen on the production of IgE antibody agree with previous results by Vaz, Vaz and Levine (1970) and Levine, Chang and Vaz (1971).

Although the mechanism of the adjuvant effect of LPS is of difficult interpretation our results suggest that the time of administration of LPS is of great importance for the preferential production of IgE antibody. Thus, while injection of LPS and antigen simultaneously resulted in a preferential production of IgE antibody, injection of LPS 24 hours after antigen stimulated the production of both IgE and IgG1. In this case, although the serum level of both antibodies was higher than when LPS and antigen were injected simultaneously there was no preferential production of IgE. This fact suggests that the induction of a preferential production of IgE antibody by LPS is restricted to a very early phase of the induction period of antibody formation when antigen first contacts antigen-sensitive cells. In this early phase, for some unknown reason, the stimulation and proliferation of IgE antibody-forming cells predominates over the IgG1 antibody-producing cells. In this regard, it is worth recalling that LPS is a mitogen specific for B-cells, being able to stimulate the synthesis of DNA by these cells but not by T-cells; besides, LPS has the interesting property of activating the proliferation of antibody-forming cells specific for a number of unrelated antigens in their absence (Sjöberg, 1971; Ortiz-Ortiz and Jaroslow, 1970; Moller, Anderson and Sjöberg, 1972). Therefore, it is possible that the efficiency of LPS in inducing the production of IgE may be due to its ability to activate the B-cells. In this case, the activation of antigen-stimulated B-cells by LPS may be more efficient for the proliferation of IgE antibody-forming cells than the activation of these cells by antigen alone. Alternatively, the preferential early synthesis of IgE antibody induced by LPS may be due to by-pass of the regulatory effect of T-cells on antibody production. Indeed, it has recently been shown that activated T-cells, in addition to their well-known ability to co-operate with B-cells can exert a suppressor effect on antibody formation (Gershon and Kondo, 1970; Gershon, Cohen, Hencin and Liebhaber, 1972) and Okumura and Tada (1971a, 1971b) have presented evidence for a regulatory effect of T-cells on IgE antibody production in rats. Further experimental data are required for an adequate interpretation of this phenomenon.

REFERENCES

Catty, D. (1969). 'Immunology of nematode infections.' *Trichinosis in Guinea Pigs as a Model*. S. Karger, Basel.

Dobson, C., Morseth, D. J. and Soulsby, E. J. L. (1971). 'Immunoglobulin E-type antibodies induced by Ascaris suum infections in guinea pigs.' *J. Immunol.*, **106**, 128.

Farthing, S.R. and Holt, L.B. (1962). 'Experiments designed to determine the mechanism of the adjuvant activity of Gram-negative organisms upon antibody production.' *J. Hyg. (Camb.)*, **60**, 44.

Gershon, R. K., Cohen, P., Hencin, R. and Liebhaber, S. A. (1972). 'Supressor T cells.' *J. Immunol.*, **106**, 619.

GERSHON, R. K. and KONDO, K. (1970). 'Cell interactions in the production of tolerance: the role of thymic lymphocytes.' *Immunology*, **18**, 721.

JOHNSON, A. G., GAINES, S. and LANDY, M. (1956). 'Studies on the antigen of *Salmonella typhosa*. V. Enhancement of antibody response to protein antibody by the purified lipopolysaccharide.' *J. exp. Med.* **103**, 225.

KABAT, E. and MAYER, M. (1961). *Experimental Immunochemistry*. Charles C. Thomas, Springfield, Ill.

KIND, P. and JOHNSON, A. G. (1959). 'Studies on the adjuvant action of bacterial endotoxins, on antibody formation. I. Time limitation of enhancing effect and restoration of antibody formation in X-irradiated rabbits.' *J. Immunol.*, **82**, 415.

LEVINE, B. B., CHANG, H. and VAZ, N. M. (1971). 'The production of hapten specific reaginic antibodies in guinea pig.' *J. Immunol.*, **106**, 29.

MERRIT, K. and JOHNSON, A. G. (1963). 'Studies on the adjuvant action of bacterial endotoxins, on antibody formation. V. The influence of endotoxin and 2-fluoro-2-deoxyuridine on the primary response of the balb mouse to a purified antigen.' *J. Immunol.*, **91**, 266.

MOLLER, G., ANDERSSON, J. and SJÖBERG, O. (1972). 'Lipopolysaccharide can convert heterologous red cells into thymus-independent antigens.' *Cell Immunol.*, **4**, 416.

MOTA, I. (1963). 'Biological characterization of mast cell sensitizing antibodies.' *Life Sciences*, **1**, 465.

MOTA, I. (1964). 'The mechanism of anaphylaxis. I. Production and biological properties of mast cell sensitizing antibody.' *Immunology*, **7**, 681.

MOTA, I. (1967). 'Biological characterization of mouse-early-antibody.' *Immunology*, **12**, 343.

MOTA, I. and PEIXOTO, J. M. (1966). 'A skin-sensitizing and thermolabile antibody in the mouse.' *Life Sciences*, **5**, 1723.

MOTA, I. and PERINI, A. (1970). 'A heat labile mercaptoethanol susceptible homocytotropic antibody in the guinea pig.' *Life Sciences*, **9**, 923.

MOTA, I., WONG, D., SADUN, E. H. and GORE, R. W. (1969). 'Mouse homocytotropic antibodies.' *Cellular and Humoral Mechanisms in Anaphylaxis and Allergy*. p. 13. S. Karger, Basel.

OKUMURA, K. and TADA, T. (1971a). 'Regulation of homocytotropic antibody formation in the rat. III. Effect of thymectomy and splenectomy.' *J. Immunol.*, **106**, 1019.

OKUMURA, K. and TADA, T. (1971b). 'Regulation of homocytotropic antibody formation in the rat. VI. Inhibitory effect of thymocytes on the homocytotropic antibody responses.' *J. Immunol.*, **107**, 1682.

ORTIZ-ORTIZ, L. and JAROSLOW, B. N. (1970). 'Enhancement by the adjuvant endotoxin of an immune response induced *in vitro*.' *Immunology*, **19**, 387.

OVARY, Z. (1964). 'Passive cutaneous anaphylaxis.' *Immunological Methods* (Ed. by J. R. A. Ackroyd). p. 259. Blackwell Scientific Publications, Oxford.

OVARY, Z., BENACERRAF, B. and BLOCH, K. L. (1963). 'Properties of guinea pig 7S antibodies. II. Identification of antibodies involved in passive cutaneous and systemic anaphylaxis.' *J. exp. Med.*, **117**, 951.

PARISH, W. E. (1970). 'Homologous serum passive cutaneous anaphylaxis in guinea-pigs mediated by two γ1, or γ1-type heat stable globulins and a non-γ1 heat labile reagin.' *J. Immunol.*, **105**, 1296.

PERINI, A. and MOTA, I. (1972). 'Heterogeneity of guinea-pig homocytotropic antibodies.' *Immunology*, **22**, 915.

SJÖBERG, O. (1971). 'Antigen-binding cells in mice immune or tolerant to *Escherichia coli* polysaccharide.' *J. exp. Med.*, **133**, 1015.

TADA, T. and OKUMURA, K. (1970). 'Regulation of homocytotropic antibody formation in the rat. I. Feed-back regulation by passively administered antibody.' *J. Immunol.*, **106**, 1002.

VAZ, N. M., VAZ, E. M. and LEVINE, B. B. (1970). 'Relationship between histocompatibility (H-2) genotype and immune responsiveness to low doses of ovalbumin in the mouse.' *J. Immunol.*, **104**, 1572.

WESTPHAL, O. and LUDERITZ, O. (1954). 'Chemische erforschung von lipopolysacchariden gramnegativer bacterian.' *Angew. Chem.*, **66**, 107.

ZVAIFLER, N. J. and BECKER, E. L. (1966). 'Rabbit anaphylactic antibody.' *J. exp. Med.*, **103**, 935.