Immunology, 1964, **7**, 616.

The Immune Response to Azo-Protein Conjugates in Rabbits Unresponsive to the Protein Carriers

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(*Received 28th October* 1963)

Summary. Rabbits, made unresponsive to human serum albumin by means of antigen injections following total body X-irradiation, were immunized with sulphanil-azo conjugates of human serum albumin (HSA). Non-treated rabbits were similarly immunized with sulphanil-azo conjugates of rabbit serum albumin (RSA). Both groups reacted to the sulphanil-azo determinant, forming two types of antibodies. One cross-reacted with sulphanil-azo proteins of the homologous and two heterologous carriers; the other was specific to the conjugate of the homologous protein, although the animals were unresponsive to the homologous protein in its native state. In this respect, namely the immunological response to azo-protein conjugates, animals which are naturally tolerant to the protein carrier, and animals which were made tolerant to the protein carrier, are basically similar. The formation of antibodies specific to the homologous conjugates indicates that the hapten conjugation resulted in the formation of an antigenic determinant comprising both the haptenic group and part of the protein carrier. Upon further immunization with the hapten–protein conjugates, in rabbits tolerant to HSA, the tolerance to the protein carrier was abolished. Immunization of rabbits with sulphanil-azo RSA elicited antibodies which cross-reacted with HSA. The implication of these immune responses and reactions and their bearing on the mechanism of immune tolerance, and its breakdown, are discussed.

INTRODUCTION

Immune tolerance or specific immune unresponsiveness are, by definition, immunologically specific phenomena. Their specificity depends on the specific determinants of the relevant 'tolerogenic' antigens. What we encounter in practice is tolerance, or unresponsiveness, to specific antigenic determinants rather than to the antigen as a whole (Smith, 1961). The coupling of defined haptenic groups to tolerogenic antigens, thus adding new antigenic determinants at will, may therefore be a rewarding experimental approach to the study of the specificity of immune unresponsiveness.

The present study is based on such an approach as applied to unresponsiveness induced in adult rabbits by means of X-irradiation (Nachtigal and Feldman, 1963).

According to Landsteiner's classical tenet (Landsteiner, 1945), an animal immunized with a homologous protein conjugated to a haptenic group will respond with antibody formation against the haptenic group exclusively. The homologous 'carrier' protein is assumed to be inert as far as the specificity of the immune response is concerned. This

view has been modified recently, however, by evidence (Gell and Benacerraf, 1961; Benacerraf and Levine, 1962; Gell and Silverstein, 1962; Leskovitz, 1963) that, in delayed hypersensitivity reactions at least, the protein carrier of conjugated antigens is not an inert component. The reaction of guinea-pigs, sensitized with homologous protein conjugates, is always more intense when challenged with the homologous derivatives than when challenged with the same haptenic group conjugated to heterologous carriers. Similarly, desensitization is also more intense with homologous conjugates.

These findings led to some important conclusions. Apparently the antibody response of animals, immunized with homologous protein conjugated to a hapten, may not be directed specifically to the haptenic group as such, but rather to a larger molecular area comprising parts of the carrier protein molecule as well. Thus, the new antigenic determinant created when coupling a hapten to a protein carrier may provide a new steric configuration incorporating parts of both the hapten and the carrier molecules. Since in this case the carrier-protein displays immunogenic properties, it means that the coupling of a hapten to a homologous protein makes a part of the protein molecule unrecognizable as 'self' to the antibody-forming mechanism. Immunization with protein–hapten conjugates seemed to us, therefore, a sensitive method of comparing the discrimination between 'self' and 'non-self' in natural tolerance on the one hand, and in induced unresponsiveness on the other.

Work on these lines has been reported previously (Cinader and Dubert, 1955, 1957; Cinader and Pearce, 1958; Boyden and Sorkin, 1962). The results were, however, conflicting. The more recent report of Boyden and Sorkin claims that normal rabbits, immunized with sulphanil-azo rabbit serum albumin, responded with antibody formation to the newly introduced determinant. On the other hand, rabbits made tolerant to human serum albumin (HSA) by neonatal injections of the antigen, did not respond, after immunization with sulphanil-azo HSA, either to the carrier or to the sulphanil-azo determinant. These findings would mean that rabbits discriminate between their homologous albumin and its conjugate, as 'self' and 'non-self' respectively, but that, on the other hand, their antibody-forming system is incapable of such fine discrimination towards modifications of an *adopted* 'self'. Boyden and Sorkin interpreted their results as evidence that the tolerance of an animal to its own serum proteins on the one hand, and experimentally induced tolerance on the other, are based on entirely different mechanisms.

This very far-reaching conclusion merits re-examination, which we have conducted with our system of induced unresponsiveness.

MATERIAL AND METHODS

Antigens

Human serum albumin (HSA) was obtained from the Plasma Fractionation Institute of the local First Aid Society, where it was prepared according to Cohn's fractionation method. When subjected to immune electrophoresis and developed with homologous antiserum, the preparation showed a single precipitation band, thus demonstrating its purity.

Radioiodinated [^{131}I] HSA was obtained from the Radiochemical Centre, Amersham, England.

Rabbit serum albumin (RSA) was supplied as fraction V, by National Biochemicals Corp. of Cleveland, Ohio.

^{131}I labelled RSA was prepared according to the technique of Talmage, Baker and Akeson (1954) from the above preparation.

Gluten

A commercial preparation, made locally, was employed.

Sulphanil-azo Proteins

Five parts by weight of any of the above proteins were coupled with one part of diazotized sulphanilic acid. The protein was made up to 4 per cent in a 3·3 per cent solution of sodium carbonate. The ice-cold diazo compound was added gradually, in 2 per cent concentration, to the chilled protein solution on a magnetic stirrer, and the stirring was continued for 30 minutes in the cold. The mixture was then dialysed in a cellophane bag for 2 days against distilled water, and for 1 day against saline. The concentrations were then calculated from the original protein content, assuming no loss during the procedure.

Immune electrophoreses of the human and rabbit azo proteins, developed with homologous antisera, showed single precipitation bands, although of a higher mobility than that of the native proteins (see also Cinader and Dubert, 1957).

Rabbit Red Cells Coupled with Diazotized Sulphanilic Acid

Washed rabbit erythrocytes were formolized according to the method of Weinbach (1958), and preserved in the cold as a 10 per cent suspension in buffered saline at pH 7·2. One millilitre of this stock suspension was spun in the centrifuge, the supernatant discarded, and the sedimented cells resuspended in 3·0 ml. of carbonate–bicarbonate buffer, 0·5 M, pH 9·0, and chilled in ice. This suspension was coupled with a solution of diazotized sulphanilic acid containing about 5 mg. of the diazo compound, stirred in the cold for 15 minutes, and then washed three times with buffered saline at pH 7·2, in which a final 2 per cent suspension was made up.

Animals

Rabbits of both sexes weighing 2–3 kg. were used.

X-Ray Treatment

Rabbits were treated with 550 R. total body irradiation, as described elsewhere (Nachtigal and Feldman, 1963).

Serological Analysis

Total binding antibodies for HSA and RSA were estimated by means of the labelled antigen technique of Farr, as modified by Terres and Wolins (1961). The radio-iodinated albumins were employed in a concentration of 20 µg./ml. Sera with a high antibody content binding more than this amount of antigen per ml. were diluted appropriately in normal rabbit serum. Antibodies against the sulphanil-azo protein determinant were titrated by means of an agglutination reaction with rabbit red cells, coupled to diazotized sulphanilic acid. The cross-reactions of the native proteins, and their azo derivatives, were

analysed by means of micro gel-diffusion reactions in agar, employing the plastic template technique of Crowle (1961).

Experimental Design

Scheme of experiment (Table 1). The test animals were divided into five (unequal) groups,

TABLE 1

SCHEME OF EXPERIMENT

Day of experiment	*Group I rabbits*	*Group II rabbits*	*Group III rabbits*	*Group IV rabbits*	*Group V rabbits*
0	Irradiated 550 R.	Irradiated 550 R.	—	—	—
1, 15, 45, 59	Injected with HSA	Injected with HSA	Injected with HSA	—	—
76, 79, 83, 86, 90, 93, 97, 100	Injected with azo-HSA	—	Injected with HSA	Injected with azo-HSA	Injected with azo-RSA
140	Injected with azo-HSA	—	—	Injected with azo-HSA	Injected with azo-RSA

HSA—human serum albumin; RSA—rabbit serum albumin. The azo proteins employed: sulphanil-azo conjugates. All antigens were administered intravenously in 70 mg. doses on day 1 and in 17–20 mg. doses subsequently.

of which I and V were experimental and II, III and IV controls. Groups I and II were X-irradiated on day zero; groups III, IV and V were not irradiated. Twenty-four hours later (day 1) groups I, II and III were injected intravenously with 70 mg. of HSA in saline. Thereafter, 20 mg. booster injections were given every 14–16 days, to establish a state of solid unresponsiveness to HSA in groups I and II (irradiated) and to induce an immune response to the same antigen in group III (non-irradiated) as a control.

Ten weeks after irradiation we can assume, from previous experience, that the antibody-forming mechanism of the irradiated rabbits had recovered completely from radiation damage, except that the animals remained specifically unresponsive to the test protein (HSA) (Tables 2 and 3 up to day 66).

On day 76 an intensive course of immunization with sulphanil-azo HSA was begun of group I (animals unresponsive to HSA) and of group IV (normal rabbits). The injections, 17–20 mg. each, were given intravenously every 3–4 days, nine in all.

Group II received no such treatment and remained as a control-group unresponsive to HSA (Tables 2 and 3). Group III—normal rabbits immunized with HSA—received further HSA injections at the same rate as described for groups I and IV. Group V, the last of the hitherto untreated rabbits, underwent a simultaneous course of immunization with sulphanil-azo RSA.

RESULTS

AGGLUTINATION OF SULPHANIL-AZO RABBIT ERYTHROCYTES

Sera of rabbits immunized with sulphanil-azo HSA, after they had been made unresponsive to native HSA (group I), all agglutinated the azo-conjugated red cells. When

tested on day 107 in two-fold dilutions, the mean $\log_2$ titre found was 5 ± 2 (S.D.). The same was found for group V sera, that of normal rabbits immunized with sulphanil-azo RSA (an azo conjugate of the homologous albumin). This group reacted to a titre of 6 ± 2. Thus, both groups of rabbits responded to the sulphanil-azo protein determinant, although all these animals were unresponsive to the respective protein carriers.

Rabbits made unresponsive to HSA, but not immunized later on (Group II), as well as normal rabbits immunized with HSA (group III), did not agglutinate the azo-conjugated cells.

TABLE 2

GEL-DIFFUSION REACTIONS OF TEST AND CONTROL RABBIT SERA WITH THE ANTIGENS EMPLOYED

Group of rabbits	*Day* 42 *HSA*	*Day* 54 *HSA*	*Day* 66 *HSA*	*Day* 107 *HSA*	*Day* 107 *Azo-HSA*	*Day* 107 *Azo-RSA*	*Day* 107 *Azo-Gluten*
I	0/35	0/35	0/33	1/29	19/29	17/29	7/29
II	0/4	0/4	0/4	0/4	0/4	0/4	0/4
III	3/3	3/3	3/3	3/3	3/3	0/3	0/3
IV	—	—	—	2/2	2/2	2/2	2/2
V	—	—	—	0/16	14/16	14/16	3/16

Denominator: number of rabbits tested; Numerator: number of rabbits reacting positively.

GEL-DIFFUSION REACTIONS (Table 2)

Out of twenty-nine rabbits of group I (unresponsive to HSA and immunized with sulphanil-azo HSA) one gave a positive gel-diffusion reaction with HSA on day 107, a breakdown of its former unresponsiveness to this antigen.

Of the remaining twenty-eight unresponsive rabbits, eighteen gave precipitation bands with the homologous (sulphanil-azo HSA) antigen, sixteen gave precipitation bands with sulphanil-azo RSA, and seven with the azo conjugate of gluten.

Group V, normal rabbits immunized with the homologous RSA conjugate, reacted in gel diffusion with neither the homologous albumin nor HSA, yet fourteen out of the sixteen animals reacted both with the rabbit and the human conjugates. Three out of sixteen reacted also with the gluten conjugate.

In the control groups, the reactions were as follows: Group II, rabbits made unresponsive to HSA and then left unimmunized, were negative with all the antigens tested. Group III (normal, immunized with HSA) reacted with both HSA and azo-HSA, thus demonstrating that the conjugation, besides adding new determinants to HSA, did not drastically change its original specificity. Group IV, normal rabbits immunized with the human conjugate, reacted with all four antigens tested: HSA, HSA- and RSA-conjugates, as well as with the gluten conjugate.

These results are in accordance with those reported for the agglutination tests, demonstrating again that rabbits unresponsive to a carrier protein, whether naturally or through induction of unresponsiveness, can form antibodies towards a new antigenic determinant introduced into the carrier by conjugation.

The patterns of the gel-diffusion reactions were of special interest. The sera of group I rabbits (unresponsive to HSA) which were immunized with azo-HSA and reacted with both the human and the rabbit conjugates demonstrated a pattern of partial identity with the heterologous conjugate (Fig. 1) (Ouchterlony, 1939; Finger and Heller, 1960). The

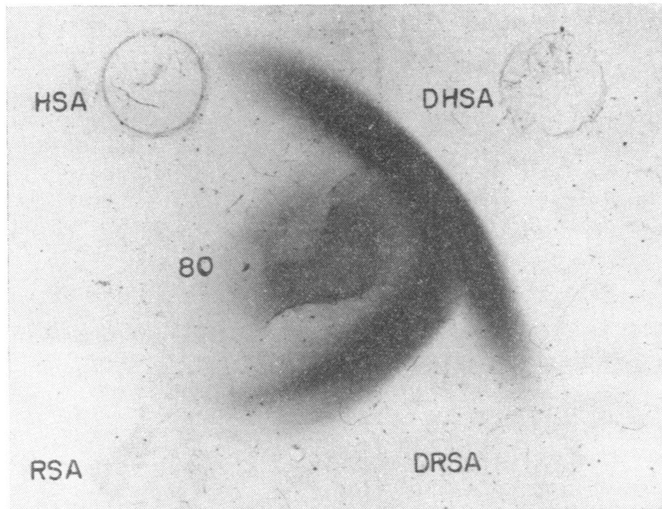

Fig. 1. Gel-diffusion reactions of serum from rabbit No. 80, unresponsive to HSA. The rabbit was immunized with azo-HSA (DHSA). The serum does not react with HSA, reacts with DHSA, cross-reacts with azo-RSA (DRSA). Note the 'spur' indicating the presence of an antigenic determinant in DHSA which is absent in DRSA.

precipitation bands testify to the presence of antigenic determinants on the homologous albumin molecule formed by the actual process of coupling. Yet the very marked spurs demonstrate that at least one determinant of the homologous conjugate is absent in the heterologous one. Conversely, rabbits of Group V, immunized with the rabbit conjugate, demonstrated that the process of coupling introduced a new antigenic determinant into the rabbit albumin, which was absent in the human conjugate (Fig. 2). These results thus

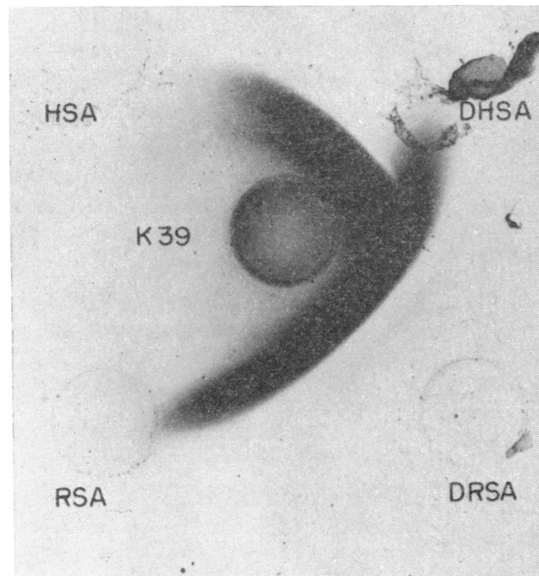

Fig. 2. Gel-diffusion reactions of rabbit No. 39, non-irradiated and immunized with DRSA. The serum reacts with DRSA and cross-reacts with DHSA. The 'spur' indicated the presence of an antigenic determinant in DRSA which is absent in DHSA.

establish the presence of 'coupling determinants' specific for each carrier protein. In addition, a less-specific anti-sulphanil-azo antibody is formed in response to each azo-protein which displays a pattern of identity even with the gluten conjugate.

We consider, therefore, that these precipitation patterns demonstrate the part played by the carrier protein in inducing the specific immune response to a hapten conjugate. These patterns exemplify the change in specificity of a part of the carrier protein molecule as a result of coupling, which introduces a 'non-self' determinant into the homologous proteins, while the unresponsiveness to the other 'self' determinants remains almost unchanged.

An attempt was made to elucidate in more detail the interplay of specificities in these gel-diffusion patterns. This was attempted by means of absorption of the anti-sulphanilic-acid antibodies through incorporation of sodium sulphanilate, or sulphanil-azo tyrosine, in the diffusion agar. These attempts were unsuccessful, probably indicating that the operative determinant in the case of sulphanil-azo proteins includes a larger area of the diazo-coupled antigen than of the hapten groupings employed (Cinader, 1957; Karush, 1962).

Another attempt to eliminate part of the antibody specificity involved, was performed as follows: rabbits were given, after X-ray treatment, a series of injections of azo-HSA in order to induce unresponsiveness. It was planned to immunize these animals later with the corresponding rabbit conjugate, in the hope of eliminating all antibodies except those directed against the specific determinants of RSA arising after coupling. The attempt failed, however, because no complete unresponsiveness could be induced to the HSA conjugate in the fifteen animals tested, under conditions which yield in our hands a solid tolerance of the native protein. All animals responded with marked antibody production to HSA, although to a lesser degree than the non-irradiated controls (Cinader and Dubert, 1957).

BINDING ANTIBODIES TO HSA AND RSA

The sera of rabbits immunized with sulphanil-azo conjugates were tested for binding antibodies to the native proteins. Neither the X-rayed rabbits treated with the HSA conjugate, nor those immunized with RSA conjugate, showed any binding capacity for rabbit serum albumin. On the other hand, both groups yielded sera that exhibited a marked capacity for binding HSA. Thus the sera of twenty-one out of twenty-nine rabbits in group I (immunized with the human conjugate) on day 107 of the experiment, bound 2 μg. or more labelled HSA per ml., i.e. over 10 per cent of the total antigen added (Table 3). When rabbits of this group were given a booster injection of the conjugate a

TABLE 3

BINDING OF HSA BY SERA OF TEST AND CONTROL RABBITS

Group of animals	*Day* 42	*Day* 54	*Day* 66	*Day* 107	*Day* 147
I	0	0	0	4 ± 4	78 ± 138
II	0	0	0	0	0
III	19 ± 1	170 ± 15	—	778 ± 228	—
IV	—	—	—	146 ± 31	540 ± 130
V	—	—	—	10 ± 13	14 ± 12

The figures denote the mean value of HSA bound, in μg. per ml. serum ± Standard Deviation (Farr's technique).

Values below 2 μg./ml. were considered as negative.

month later, a sharp rise of binding capacity for the native protein was demonstrated in their sera, amounting practically to a breakdown of the unresponsiveness to HSA (Table 3, day 147). A similar phenomenon has also been described (Cinader and Dubert, 1955; Weigle, 1962) in neonatally-induced tolerance. A totally unexpected finding, however, was the binding capacity for HSA exhibited by the sera of rabbits in group V, immunized with the rabbit conjugate. All these sera, taken on day 107, bound HSA in amounts of 2 μg. per ml. or more (normal rabbit serum served as a zero 'blank'). They showed a slight rise in titre when rechallenged a month later (Table 3).

A control experiment was performed in which rabbits made unresponsive to HSA by X-ray treatment were immunized with the RSA conjugate. In this group a good response to the azo RSA was found, while no binding antibodies to HSA were present. These findings imply that the steric changes in RSA, concomitant with diazo coupling, 'opened up' antigenic determinants either identical or overlapping with some of those present on the HSA molecule. The converse situation did not occur: diazo coupling of HSA did not disclose the emergence of determinants cross-reacting with RSA.

DISCUSSION

It has been suggested by Gell and Silverstein (1962) that the 'antibody' responsible for delayed hypersensitivity reactions in guinea-pigs is complementary to a larger area of the antigen molecule than is circulating antibody. This they deduced from their experiments with conjugated antigens, where they demonstrated that the specificity of delayed hypersensitivity reactions depended both on the haptenic group and the protein carrier. On the other hand, the specificities of the Arthus phenomenon, passive cutaneous anaphylaxis and gel-diffusion reaction, depended, apparently, on the haptenic group alone. The gel-diffusion patterns described in the present study provide evidence that the specificity of rabbit precipitins may well depend, in a like manner, on both the haptenic group and the protein carrier molecule. It remains to be seen whether this is a peculiarity of guinea-pig cell-bound antibodies and rabbit precipitins, or whether it is a general characteristic of antibodies which may not always be easy to demonstrate.

It is also evident from the patterns that conjugation to a hapten results in the appearance of at least two kinds of new antigenic determinants. One, of lesser specificity, showed cross-reactions with all the sulphanil-azo conjugates tested. The other determinant, although demonstrably specific for the carrier protein, was recognizable as not 'self' by the animal; this reflects the high degree of discrimination of the 'self' recognition mechanism. Rabbits made unresponsive to HSA and then immunized with its conjugate, behaved in this respect identically to normal rabbits immunized with the rabbit conjugate. These findings may be considered as evidence of the high degree of specificity of our system of induced unresponsiveness.

The conclusions of Boyden and Sorkin (1962) are essentially different from our own. In our study groups of rabbits immunized with a conjugate of native albumin, and those immunized with a conjugate to a protein to which tolerance was acquired, reacted in a similar manner. Both displayed antibody formation to the modified protein, but not to the native protein. When examined in more detail, however, differences were observed between the two groups of test animals.

The natural tolerance of rabbits to their homologous albumin was apparently more

solid than the acquired unresponsiveness to the human protein, which became upset at the end of the experiment. In our experience rabbits, given comparatively smaller doses of HSA after irradiation, displayed a far more durable unresponsiveness to challenge with the unmodified antigen. It must therefore be assumed that boosting with a modified determinant brought about weakening of unresponsiveness to the native protein.

However, this may not necessarily reflect inherent differences between systems of natural and induced unresponsiveness. If immunological unresponsiveness depends on the constant presence of antigen in contact with the antibody-forming apparatus, then the two groups of rabbits, I and V in our experiment, were not exactly comparable in this respect. While the naturally tolerant rabbits carried a large excess of native rabbit albumin in their circulation, the supply of HSA to rabbits with induced unresponsiveness to this protein had been discontinued before immunization with azo-HSA was started.

Although there is evidence of cross-reacting determinants in the albumins of many mammals (Weigle, 1961), the formation of anti-HSA antibodies by rabbits immunized with sulphanil azo RSA was most unexpected. It seems unlikely that cross-reacting interspecies determinants would be created by mild chemical handling of an antigen. A more reasonable assumption is that the determinant in question had been present structurally in the native rabbit albumin, although in a non-operative form due to some steric hindrance. The latent determinant might have been 'unmasked' subsequently by the diazo-coupling procedure. This observation should be considered therefore as demonstrating a latent cross-reactivity between HSA and RSA. Although it may seem to be a most unusual finding, it is in perfect agreement with the accepted views on immune tolerance, since an animal cannot be immunologically tolerant to its own masked determinants.

The existence of masked antigenic determinants in homologous proteins, as strongly suggested by the above findings, merits a further comment. Theoretically, the situation in this case may be analogous to that of 'sequestered' or 'inaccessible' antigens. These antigens are believed to bring about auto-immune disorders when the anatomical barrier which isolates them from the antibody-forming apparatus fails for some reason. Masked determinants, if they exist, might be sequestered determinants on the molecular level, which are liable to become immunogenic either because of the breakdown of a steric barrier or by a completion of a necessary chain length.

ACKNOWLEDGMENT

We are grateful to Mr. Z. Gedassy for his skilful technical assistance.

This work was supported by a grant No. C-6165 from the National Institute of Health, United States Public Health Service, Bethesda, and a National Institute of Health Agreement No. 335105.

REFERENCES

Benacerraf, B. and Levine, B. B. (1962). 'Immunological specificity of delayed and immediate hypersensitivity reactions.' *J. exp. Med.*, **115**, 1023.

Boyden, S. V. and Sorkin, E. (1962). 'Effect of neonatal injections of protein on the immune response to protein–hapten complexes.' *Immunology*, **5**, 370.

Cinader, B. (1957). 'Antibodies against enzymes.' *Annu. Rev. Microbiol.*, **11**, 371.

Cinader, B. and Dubert, J. M. (1955). 'Acquired immune tolerance to human albumin and the response to subsequent injections of diazo human albumin.' *Brit. J. exp. Path.*, **36**, 515.

Cinader, B. and Dubert, J. M. (1957). 'Specific inhibition of response to purified protein antigens.' *Proc. roy. Soc.* B, **146**, 18.

Cinader, B. and Pearce, J. H. (1958). 'The specificity of acquired immunological tolerance to azo proteins.' *Brit. J. exp. Path.*, **39**, 8.

Crowle, A. J. (1961). *Immunodiffusion*, p. 204. Academic Press, New York.

Finger, I. and Heller, C. (1960). 'Gel diffusion analysis of cross-reactions of a protein–hapten conjugate.' *J. Immunol.*, **85**, 332.

Gell, P. G. H. and Benacerraf, B. (1961). 'Studies on hypersensitivity. IV. The relation between contact and delayed hypersensitivity. A study on the specificity of cellular immune reactions.' *J. exp. Med.*, **113**, 471.

Gell, P. G. H. and Silverstein, A. M. (1962). 'Delayed hypersensitivity to hapten–protein conjugates.' *J. exp. Med.*, **115**, 1037.

Karush, F. (1962). 'Immunological specificity and molecular structure.' *Advanc. Immunol.*, **2**, 6.

Landsteiner, K. (1945). *The Specificity of Serological Reactions*, p. 158. Harvard University Press, Cambridge, Mass.

Leskovitz, S. (1963). 'Use of oxidized proteins in the examination of immunochemical specificity in delayed hypersensitivity to hapten–protein conjugates.' *Nature (Lond.)*, **199**, 85.

Nachtigal, D. and Feldman, M. (1963). 'Immunological unresponsiveness to protein antigen in rabbits exposed to X-irradiation or 6-mercaptopurine treatment.' *Immunology*, **6**, 356.

Ouchterlony, Ö. (1939). 'Antigen–antibody reactions in gels.' *Acta path. microbiol. scand.*, **32**, 231.

Smith, R. T. (1961). 'Immunological tolerance of nonliving antigens.' *Advanc. Immunol.*, **1**, 104.

Talmage, D. W., Baker, H. R. and Akeson, W. (1954). 'The separation and analysis of labelled antibodies.' *J. infect. Dis.*, **94**, 199.

Terres, G. and Wolins, W. (1961). 'Enhanced immunological sensitization of mice by the simultaneous injection of antigen and specific antiserum.' *J. Immunol.*, **86**, 361.

Weigle, W. O. (1961). 'Immunological properties of the cross-reactions between anti-BSA and heterologous albumins.' *J. Immunol.*, **87**, 599.

Weigle, W. O. (1962). 'Termination of acquired immunological tolerance to protein antigens, following immunization with altered protein antigens.' *J. exp. Med.*, **116**, 913.

Weinbach, R. (1958). 'Die Verwendbarkeit formolbehandelter Erythrocyten als Antigentraeger in der indirekten Haemagglutination.' *Schweiz. Z. allg. Path.*, **21**, 1043.