THE IMMUNE RESPONSE TO A HYBRID PROTEIN MOLECULE

Specificity of Secondary Stimulation and of Tolerance Induction*

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(Received for publication 16 May 1967)

When an antigen is injected into a higher animal, a sequence of reactions occurs which culminate in the manifestations of immunity, e.g., antibody formation. A number of experimental approaches are available for obtaining information on this sequence of reactions. Specificity studies on the process of tolerance induction, on the one hand, and on secondary stimulation on the other, carried out by using pairs of well-defined cross-reacting antigens have revealed phenomena which are probably connected with the early phase of the immune response. Tolerance to an antigen could be overcome by injecting antigens which cross-reacted with the "tolerogen" (1–9); several workers have demonstrated that an antigen may stimulate the production of antibodies directed to another though related antigen with which the organism had previously been in contact (10–15). Both the overcoming of tolerance and the phenomenon of "original antigenic sin" (16) have been explained (5, 9, 14) by postulating certain receptors for antigen whose specificity did not necessarily seem to be identical with the specificity of the antibodies produced. Experimental tolerance would be induced on the level of these receptors rather than on the level of antibody production. On the basis of these ideas, the finding of a broader specificity of tolerance than of the antibodies produced (1, 4, 5, 17) could equally well be explained as the existence of cross-tolerance of serologically non-cross-reacting antigens (18).

Our studies were undertaken to define, in a suitable antigen system, the specificities of both tolerance induction and the induction of the secondary response. The system of lactic dehydrogenase (LDH) isoenzymes appears especially promising for such specificity studies (for review see Kaplan, reference 19). It consists of five tetrameric enzyme proteins which are composed of two types of subunits, A and B, in all possible combinations. Subunit A and B (in their tetrameric form) do not cross-react serologically (20, 21, 22). The following

* This study was supported by the Deutsche Forschungsgemeinschaft and by the Stiftung Volkswagenwerk.

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LDH antigens were chosen for our studies: LDH-I, subunit composition BBBB; LDH-III, subunit composition BBAA; LDH-V, subunit composition AAAA.[1] The characteristic features of this antigen system are obvious: It allows for arranging two sets of antigenic determinants either on the same or on two separate molecules. It does not require the use of carrier molecules, nor should combining both types of determinants change their serological properties.

The results of our studies show that subunit A plays a predominant role in the process of tolerance induction to LDH-III and in the stimulation of a secondary response to the enzyme, whereas the antibody population induced by LDH-III consists of approximately equal amounts of anti-A and anti-B antibodies.

Materials and Methods

Enzymes.—

LDH-I: Crystalline LDH from pig heart was prepared essentially as described by Wachsmuth and Pfleiderer (23). Since the enzyme is stable in 2 M ammonium sulphate at 75°C, the protein fraction precipitated from the crude extract by ammonium sulphate (70% saturation) was subjected to these conditions for 5 min. The mixture was then centrifuged, and the enzyme was crystallized from the supernatant by addition of ammonium sulphate. It was then purified further by ammonium sulphate fractionation up to a specific activity of 18,000–22,000 Bücher units. The yield was 3–6 g from 14 kg heart tissue. From this preparation, LDH-I was isolated by passage through a column of DEAE-Sephadex A50 (Pharmacia, Uppsala, Sweden) equilibrated with 0.01 M phosphate buffer, pH 7.2, containing 0.2 M NaCl. Under these conditions, LDH-II, -III, -IV, and -V were not adsorbed to the exchanger, whereas LDH-I passed the column very slowly. LDH-I was crystallized from the eluate by addition of solid ammonium sulphate and recrystallized once from 0.067 M phosphate buffer. The yield was 1.5-2 g of 3 g LDH applied to the column. In most cases, the enzyme was rechromatographed in the same way. All enzyme preparations used had specific activities of 20,000–24,000 Bücher units and were electrophoretically homogeneous (contamination with hybrid LDH enzymes <0.1 ‰). In passive hemagglutination inhibition tests, the enzyme was shown to contain 0.1‰ or less of A subunits after a single chromatography.

LDH-V: LDH from porcine skeletal muscle was prepared as described by Jécsai (24) or obtained from Boehringer und Soehne GmbH., Mannheim, Germany. The preparations had specific activities of 30,000–40,000 Bücher units. They contained LDH-V very predominantly. To eliminate the contaminating hybrid enzymes, each enzyme preparation was passed through a column of DEAE-Sephadex A50 equilibrated with 0.01 M phosphate buffer, pH 7.2 (25). Under these conditions, LDH-V passes the column, whereas all hybrid enzymes are adsorbed to the exchanger. Since, in the case of LDH-V, a very high degree of purity is required (see Results), the enzyme preparations were chromatographed twice. They then had specific activities of 35,000–40,000 Bücher units and were homogeneous in electrophoresis (a contamination of 1 ‰ being detectable). By passive hemagglutination inhibition, the enzyme was shown to contain less than 5×10^{-5} w/w contamination of B-specific material.

Porcine LDH-III was obtained from Boehringer. This enzyme had been prepared by in vitro hybridization of LDH-I and LDH-V according to the conditions described by Markert (26). The enzyme was crystalline, the specific activity being 28,000 Bücher units. By analysis

[1] LDH-I and LDH-V are occasionally referred to as "pure forms," as opposed to the hybrid enzyme LDH-III.

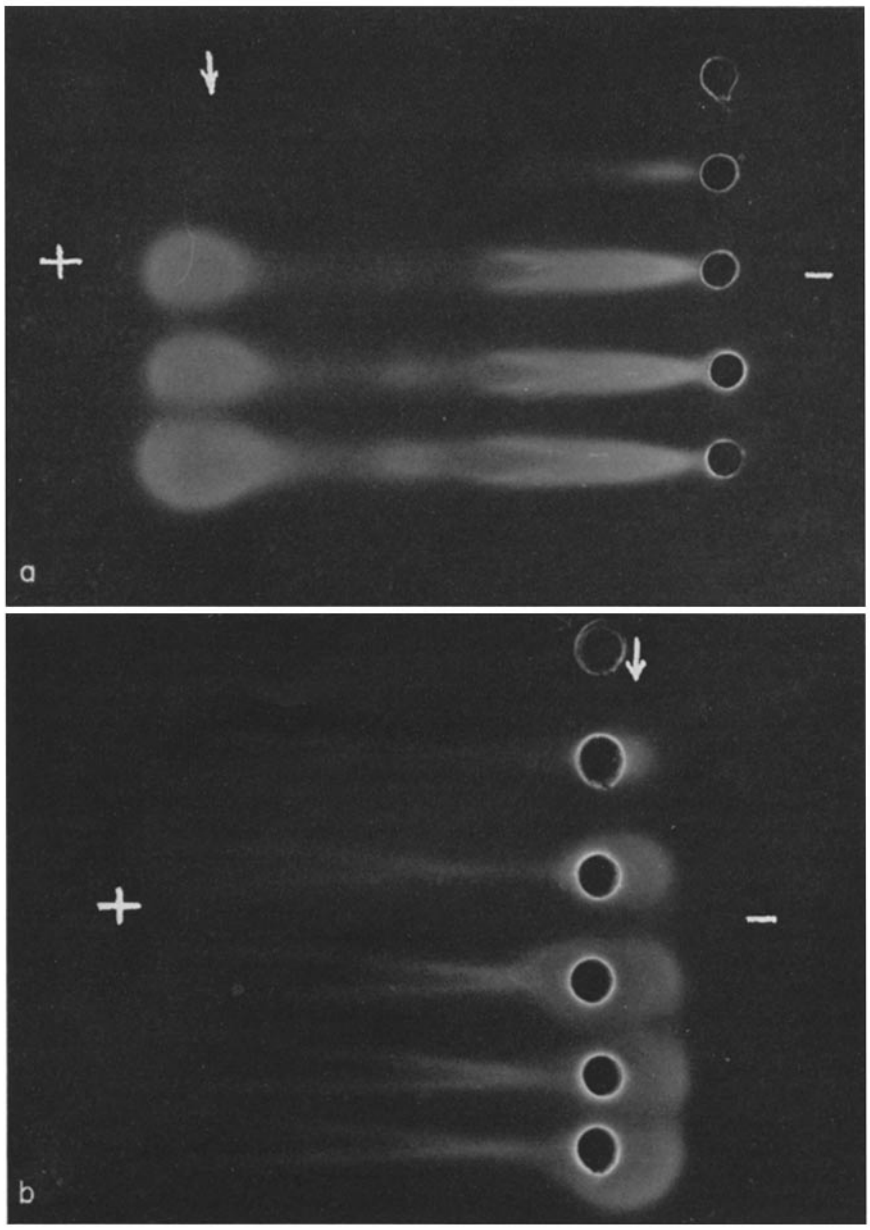

FIG. 1. Titration of antisera to LDH-I (*a*) and LDH-V (*b*) with the homologous enzyme antigens. Agarose electrophoresis and subsequent LDH-specific staining. The arrows indicate the typical position of the free enzyme after electrophoresis. In both cases, free enzyme is detected from the second sample on (first sample on top). The smear of enzymatically active material from the reservoir towards the anode is thought to be due to soluble antigen antibody complexes present in the reaction mixtures.

in agar electrophoresis, the preparation was shown to contain approximately 1% of LDH-II and LDH-IV each, and <0.1% of the pure forms.

Antibody Titration.— The sera of immunized animals were titrated in the following way (27): To constant amounts of antiserum (0.025 ml) increasing amounts of enzyme antigen were added, and the volume of the reaction mixture was adjusted to 0.125 ml with saline (for low enzyme concentrations 0.1 ml of serum was taken, the final volume again being 0.125 ml). After mixing and incubating for 1 hr at 37°C and 1 hr at room temperature, the samples were subjected to agarose electrophoresis with subsequent LDH-specific staining. Fig. 1 shows the typical patterns obtained with LDH-I and LDH-V and homologous antisera. The equivalence point of a serum was associated with an enzyme concentration which just permits the detection of a trace of free enzyme in the supernatant. It was shown by quantitative precipitation that, at this enzyme concentration, maximal precipitation occurs in homologous LDH-anti-LDH systems. In heterologous systems, however, the cross-reacting antibodies often precipitate the antigen poorly though combining with it. In such a situation, the titration method gives more relevant results than quantitative precipitation: In a more or less broad dose range, soluble antigen antibody complexes, but no free enzyme, appear in the supernatant upon electrophoresis. The titration method as described has the following advantages: (*a*) it is done rapidly and is very reproducible. (*b*) It is absolutely specific for the enzyme antigen used. It proved especially useful in that it made possible the titration of an antiserum, in one and the same series of reaction mixtures with both LDH-I and LDH-V, because of the different electrophoretic mobilities of these enzymes. (*c*) It gives a useful approximation of the antibody content of an antiserum. (*d*) It is sensitive enough for many purposes. Equivalence points of 0.1 μg per ml serum for LDH-I and 1.0 μg per ml serum for LDH-V can be determined. In this dose range, however, only a very approximate estimation of the equivalence point can be made because of the limited sensitivity of the LDH-specific stain. Figs. 2 and 7 show how precisely equivalence points can be determined by routine titration in the various dose ranges. If necessary, a precision approximately two times higher can be achieved.

Passive Hemagglutination.—Coupling of LDH-III to pigeon red cells was performed as described by Kabat and Mayer (28) with the following modifications: The incubation mixture contained 1 ml of LDH-III (2 mg/ml in saline), 1 ml of bovine serum albumin (BSA) (2 mg/ml in saline), 0.3 ml of washed and packed pigeon red cells, 3.5 ml of 0.15 M phosphate buffer pH 7.2, 5.1 ml saline, and 0.5 ml bis diazotized benzidine. The use of pigeon red cells instead of the commonly used sheep red cells resulted in a somewhat higher sensitivity of the test and a markedly better reproducibility from one red cell batch to the other. Also, because of the rapid sedimentation of the pigeon red cells, the test could be read after a very short incubation time (15 min). Bovine serum albumin (Behring Werke, Marburg/Lahn, Germany) was used to prevent lysis of the red cells. Bis diazotized benzidine was prepared (28), stored at —70°C, and diluted 1:10 immediately before use.

The sensitized red cells were washed twice and resuspended in saline. The passive hemagglutination test was carried out conventionally, except that 10% normal rabbit serum was used for antiserum dilutions. The microtiter equipment we used was obtained from Cooke Engineering Co., Alexandria, Va. Results were recorded as +, ±, and −.

Passive Hemagglutination Inhibition.—This was performed by diluting the antiserum in 10% normal rabbit serum containing various concentrations of inhibitor. Sensitized red blood cells were added 15–30 min later. Tests were always run in duplicate. The figures show the decrease of hemagglutination titer (expressed as per cent of the reciprocal highest serum dilution giving a positive test without inhibitor; logarithmic scale) plotted vs. the inhibitor concentration (referred to as concentration per ml of the antiserum dilutions; linear scale). As theoretically expected, straight lines were generally obtained over a wide dose range in a double logarithmic plot for the homologous system (i.e., inhibition by LDH-III of the agglutination of LDH-III-coated erythrocytes by antisera to LDH-III). This was, however,

not true for the heterologous inhibitors (LDH-I and LDH-V). A double logarithmic plot was therefore not chosen.

Separation of Heavy and Light Antibodies by Sucrose Gradient Centrifugation.—A linear sucrose gradient (29) ranging from 10%–37% w/v sucrose was prepared in phosphate buffered saline, pH 7.2. 0.01–0.5 ml antiserum and 0.03 ml of a solution of beef liver catalase (Boehringer) in saline (2 mg/ml) were layered on the top of the gradient. The total volume was 5.3 ml. Centrifugation was done at 0°C in a Spinco model L ultracentrifuge for 16 hr at 35,000 rpm. using an SW 39 swinging bucket rotor. The gradient was then divided into 12–24 fractions, and antibody activity was determined by passive hemagglutination as described above. The position of the catalase was checked in the microplates used for hemagglutination by addition of 0.025 ml of 1% H_2O_2. Since the enzyme has a sedimentation constant of 11.15s (30), it is situated in the gradient between the peaks of 19s and 7s antibodies.

The other biochemical and immunochemical methods used (determination of protein, LDH activity test, LDH-specific staining, agarose electrophoresis, double diffusion, quantitative precipitation, specific purification of anti-LDH antibody, acetylation of LDH-I) have been described (27).

Animals.—Wild type rabbits, supplied by a local breeder, were used throughout.

Standard Immunization.—The animals were primed by injecting the antigen together with Freund's complete adjuvant (Difco Laboratories Detroit, Mich.). To this end the stock suspension of the respective LDH-antigen was diluted with 0.15 M NaCl to a concentration of 2 mg/ml and cleared by centrifugation. The resulting solution contained considerable amounts of ammonium sulphate (0.15–0.8 M), since LDH is kept as a suspension in 0.067 M phosphate buffer containing 2 M ammonium sulphate. Since ammonium sulphate stabilizes the enzyme and also prevents hybridization of LDH-I and LDH-V, the solution was not dialyzed but was directly emulsified with an equal part of Freund's complete adjuvant. Great care was taken to assure a homogeneous emulsification. 1.5 ml of the emulsion was then equally distributed among the four footpads of the right hind leg. After an interval of 3.5–4 wk, the animals were boosted by intravenous injection of 1.5 mg of antigen without adjuvant. In the course of the work, it was found that an especially regular secondary response was obtained by giving two intravenous injections, the first consisting of 1.0 mg of antigen, the second 2 days later of 1.5 mg. Peak antibody titers were reached 7 days after the first injection.

Induction of Tolerance.—To induce tolerance, rabbits were injected with antigen intraperitoneally within the first 24 hr of life. Control animals received saline containing 0.02 M ammonium sulphate. Each single litter consisted of experimental animals and controls. Most experiments were done twice, completely independently of each other (see Results). Great care was taken to acertain the homogeneity of the enzyme preparations used. Though each stock preparation had already been subjected to rigid criteria for purity (see above), the following precautions were taken in addition. After dialysis against 0.15 M NaCl buffered with 0.067 M phosphate buffer, pH 7.2, the specific catalytic activity was determined. The dialyzed enzyme was stored at most for 48 hr at 4°C. There is no loss of activity during that time. Immediately before injection, the solution was centrifuged for 10 min at 5000 rpm. The animals were then injected, and the rest of the enzyme solution was analyzed by electrophoresis with subsequent LDH-specific staining for the presence of any contamination with other LDH isoenzymes. Such a contamination was in no case detected.

Statistical Analysis.—The statistical evaluation of the data was based on rank correlation of the titers observed in individual animals. The U test of Mann and Whitney as described by Lienert (31) was used. The null hypothesis was rejected if $P < 0.05$.

RESULTS

The Standard Immunization.—Like certain serum proteins, LDH isoenzymes prove to be poor immunogens if injected intravenously into the rabbit. After a

priming injection of LDH incorporated in complete Freund's adjuvant, however, the animals acquire the capacity to respond to an intravenous injection of the antigen with a rapid increase of the titer of circulating antibody, typical of a secondary response.

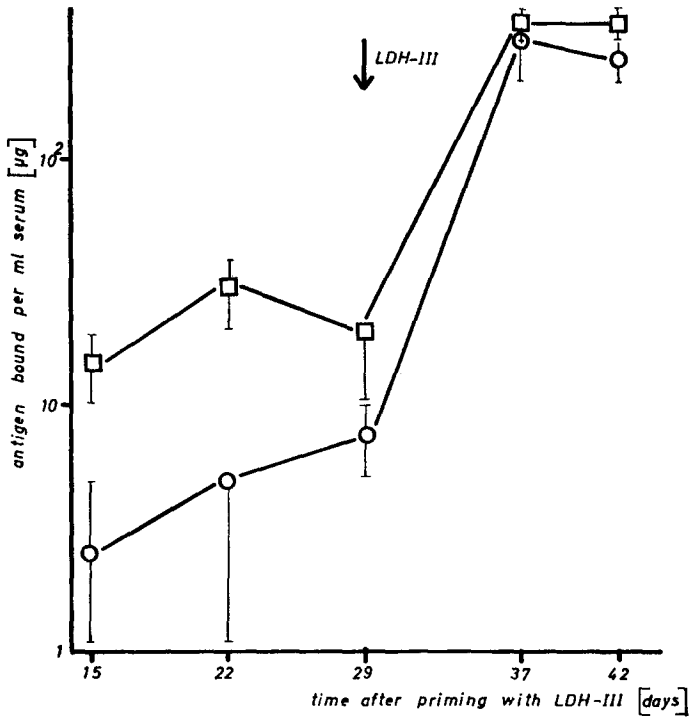

FIG. 2. Standard immunization with LDH-III. The response of a representative rabbit is shown. O—O, anti-I titers; □—□, anti-V titers. Upper and lower limits indicate definite excess of antigen and of antibody respectively. Absence of an upper limit means that at the given equivalence point just a trace of free enzyme appeared in the supernatant.

The time course of such an immunization with LDH-III is given in Fig. 2. As can be seen from the diagram, the animal in question produced, in comparable amounts, antibodies reacting with LDH-I and LDH-V. The immune response shown in Fig. 2 is very regular and reproducible. In nearly all animals, considerable amounts of antibody are formed at the height of the secondary response. And no rabbit has been found which formed predominantly anti-I or anti-V antibodies on immunization with LDH-III; the ratio of anti-I to anti-V antibodies is kept rather constant. The relevant data are contained in Fig. 3 (for the correlation of anti-I and anti-V titers in individual animals see Fig. 9). In general, rabbits with high secondary titers also showed a strong primary reac-

tion, whereas in poor responders both primary and secondary antibody titers were low. Furthermore, since the maximum of the primary response is not easily found and high antibody titers can be determined more precisely than low ones, peak secondary titers were taken as an expression of the reactivity of an animal towards LDH-III and were used in this sense for the calculations. The primary reaction was, however, always followed in the animals. Fig. 3 also contains the data obtained upon immunization with LDH-I and LDH-V. It appears that LDH-I, especially, is a rather irregular immunogen in rabbits.

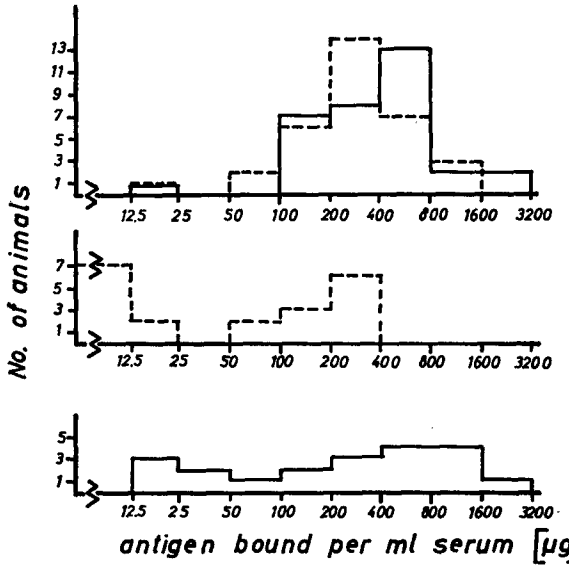

FIG. 3. The immune response of rabbits to porcine LDH-III (top), LDH-I (middle), and LDH-V (bottom). Animals were classified according to peak secondary titers (abscissa). Dashed lines, anti-I titers; unbroken lines, anti-V titers. The diagram contains data of various independent experiments which yielded similar results.

According to the extent of the response to that enzyme, our animal population seems to contain two groups of animals.

Immunochemical Characterization of the System.—

Types of antibody in standard antisera to LDH: Standard antisera to LDH were analyzed for the presence of light and heavy antibody molecules by sucrose gradient centrifugation. It was found (Fig. 4) that antibody activity was mainly associated with 7S IgG in its typical position. This activity was not destroyed by 0.2 M mercaptoethanol. A minor peak of mercaptoethanol-sensitive antibody of the 19S type, very probably IgM, was also present in most antisera analyzed. In primary antisera, this peak was sometimes as large as the peak of IgG.

Previous specificity studies in the LDH system (20–22) have been carried out by using secondary antisera and thus depend largely on the specificity of IgG antibodies. In these studies, LDH-I and LDH-V were found to be serologically unrelated. Based on the analysis of many individual antisera by titration, it can be stated that if there exists a cross-reaction between the two enzymes, it must be <0.05 % for secondary antisera to LDH-V and <0.03% for secondary antisera to LDH-I. It was important to know whether 19S antibodies to LDH distinguish, as clearly as IgG antibodies, between subunits A and B. We have previously shown (27, 32) that subunits A and B indeed possess similar surface areas which, however, are not recognized as antigenic determinants, at least as far as 7S antibodies are concerned. It was found (unpublished observations) that 19S anti-V antibody reacts poorly, if at all, with LDH-I, and vice

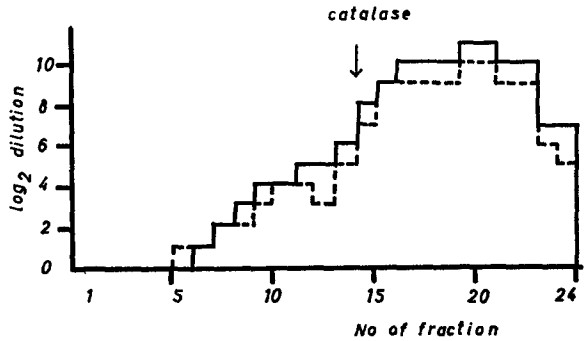

FIG. 4. Separation by sucrose gradient centrifugation of antibodies present in a pool of secondary antisera to LDH-III. The activity of the fractions obtained was tested by passive hemagglutination, using erythrocytes coated with LDH-I (dashed lines) or LDH-V (unbroken lines). The antibody activity in fractions 5–11 was completely destroyed by treatment with 0.2 M mercaptoethanol (1 hr at 37°C), whereas the activity of the main peak persisted.

versa. A low degree of cross-reactivity, however, could exist. This problem is the subject of further investigation.

Precipitation of antibodies to LDH-III by LDH-I and LDH-V: The amounts of precipitating antibody in antisera to LDH-III that react with LDH-I, LDH-III, and LDH-V were determined by quantitative precipitin analysis. The results obtained for four pools of antisera are given in Table I. It is clear from the data that similar amounts of protein can be precipitated from the sera by addition of LDH-III and by successive reaction of the sera with LDH-I and LDH-V.

The absence in antisera to LDH-III of antibodies reacting with both LDH-I and -V: If LDH-I and -V are compared in double diffusion by antisera to LDH-III, clear-cut reactions of nonidentity are obtained (Fig. 5). A similar result was obtained by titrating the antisera for anti-I and anti-V antibody: Our titration method permits an antiserum to be titrated with LDH-I and -V in two ways: either by running two separate titration series or by adding both enzymes to the same serum sample(s) (see Materials and Methods). Four pools of antisera to LDH-III were repeatedly titrated in parellel by both ways. In all cases, the same solutions of the diluted enzymes were used for both tests. Consistently, identical titers were obtained in these titrations.

These experiments demonstrate that the majority of the antibodies present in antisera to LDH-III react only with one type of subunit and not with the other. They do not exclude, however, the presence in the antisera of up to 10% of cross-reacting antibodies. Indeed we found that the amount of protein precipitated from an anti-III antiserum by one of the pure forms depended to some extent on whether the antiserum had been previously absorbed by the other pure form. The differences observed varied from 0 – 15% of the total protein precipitated. They did not depend on the presence or absence of complement.

Is this effect due to the existence of antibodies reacting with both LDH-I and -V? A variety of individual antisera and pools of antisera to LDH-III were absorbed at equivalence by LDH-I or -V respectively, and the antibodies were dissociated from the washed precipitates as previously described (27). The antibodies were then

TABLE I

Quantitative Precipitation of Secondary Antibody to LDH-III

Antiserum	Antibody precipitated by		
	LDH-I*	LDH-V*	LDH-III*
	mg/ml serum	*mg/ml serum*	*mg/ml serum*
Pool A	2.03 ± 0.03‡	2.23 ± 0.06	4.32 ± 0.13
Pool B	1.83 ± 0.06	2.4 ± 0.1	4.20 ± 0.15
Pool C	1.47 ± 0.06	1.89 ± 0.06	3.3 ± 0.2
Pool D	1.65 ± 0.06	1.71 ± 0.07	3.2 ± 0.2

* Precipitations with LDH-I and LDH-III were done using the complete and noninactivated antisera, whereas for precipitation with LDH-V the antisera had previously been adsorbed at equivalence with LDH-I.

‡ The calculation of the absolute errors was based on the evaluation of two extreme curves which could be fitted to the experimentally determined points.

titrated with LDH-I and -V. Consistently, small amounts of antibodies reacting with the pure form not used for absorption were detected in the preparations, the equivalence point being 10–20 times lower than the one for the "right" enzyme.

Since the LDH-I and -V preparations used in these tests were virtually free of any contamination with hybrid LDH enzymes (see Materials and Methods), this effect could only be due to the existence of cross-reacting antibodies or to coprecipitation of antibody not depending on a conventional serological similarity of LDH-I and LDH-V. The latter possibility was suggested by the fact that LDH-I and LDH-V showed clear-cut reactions of nonidentity when tested with our antibody preparations in double diffusion. To clarify the point further, antibodies were precipitated from a large pool of anti-III sera by LDH-V and purified. The preparation had a concentration of 40 mg of antibody protein per milliliter. The equivalence points for LDH-V and LDH-I were determined as 9500 μg and 400 μg respectively. The antibodies reacting with LDH-I were precipitated by that enzyme and again purified. As shown by analysis in double diffusion and by titration, the resulting antibody preparation consisted of nearly equal amounts of anti-I and anti-V antibodies. Again no cross-

reacting antibodies could be detected. It is assumed, therefore, that there exists very little, if any, antibody reacting with both LDH-I and -V in antisera to LDH-III.

Is antibody to LDH-III as well adapted to the hybrid enzyme as to the pure forms? Difficulties arise if one tries to compare, on the basis of the precipitin reaction, the fitting of anti-III antibodies to LDH-III, on the one hand, and LDH-I and -V on the other. These difficulties come from the fact that the hybrid molecule can carry on its surface, at most, half as many determinants of either pure form as the pure forms carry themselves. Thus, an antibody to the B subunit induced by LDH-III may fit much better to the hybrid enzyme than to LDH-I but could more readily precipitate

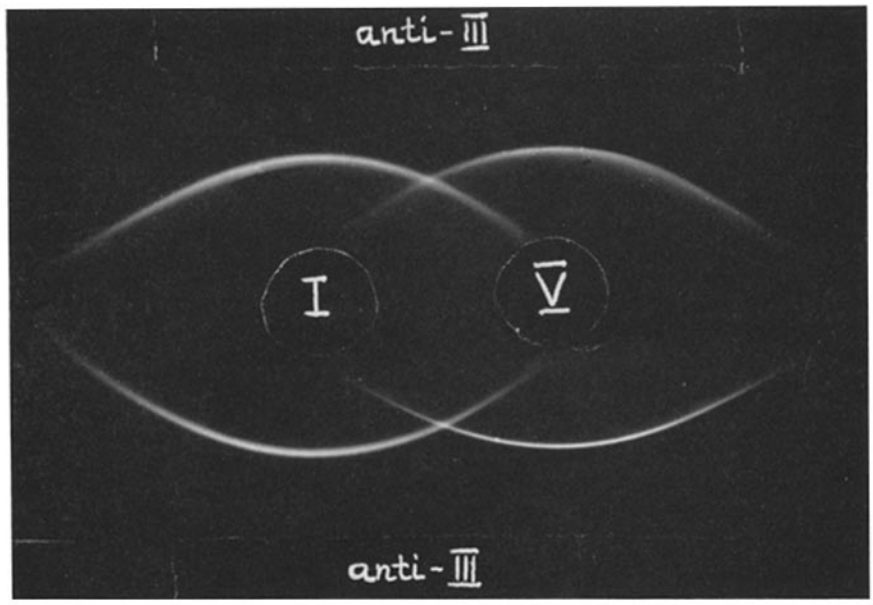

FIG. 5. Comparison in double diffusion of LDH-I and LDH-V, using a pool of secondary antisera to LDH-III.

LDH-I because the density of determinants is higher on that molecule. It may suffice to say here, therefore, that antibodies provoked by LDH-III revealed strong similarities between LDH-III, on the one hand, and LDH-I and LDH-V, on the other, in precipitin tests (i.e., quantitative precipitin analysis and double diffusion).

More satisfying results were obtained when passive hemagglutination inhibition (1) was used to test the specificity of the antibodies.[2] The conditions of the test were chosen to permit, as much as possible, competition for the antibody of the antigen fixed onto the surface of erythrocytes and antigen in solution. Fig. 6 shows the behavior of anti-A and anti-B antibodies from representative pools of antisera to

[2] We are indebted to Dr. B. Cinader of the University of Toronto for suggesting the use of this technique.

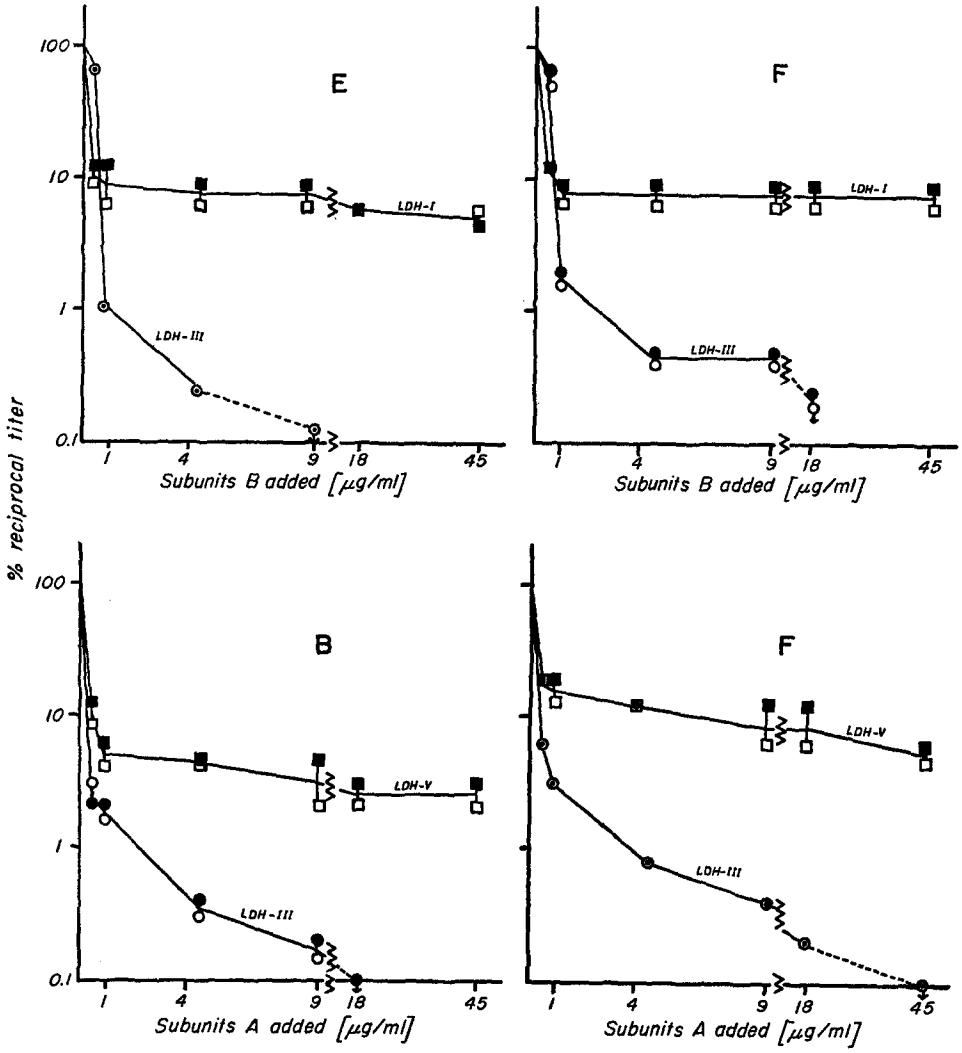

FIG. 6. Specificity of antibody in pooled secondary antisera to LDH-III. Pools E and F (top) were adsorbed by LDH-V, pools B and F (bottom) by LDH-I. Open and filled circles and squares represent independent experiments. The inhibitor concentration was converted to the concentration in the reaction mixture of subunit B and A respectively. The first serum dilution in the plates was 1:16.

LDH-III. It is evident that the majority of the antibodies to the hybrid cannot distinguish, under the conditions of the test, between LDH-III and the respective pure form. The test also reveals the existence of a small population of anti-III antibodies which seem to be hybrid-specific. We have shown that these antibodies can neither

be inhibited by a large excess of the respective pure form (up to 900 μg/ml of the reaction mixture) nor by a mixture of LDH-I and LDH-V. As demonstrated in a parallel paper (33), however, a nearly complete inhibition is obtained if one uses an inhibitor composed of two porcine subunits B and two rabbit subunits A. Since this enzyme had been prepared in our laboratory from highly purified batches of LDH-I and LDH-V, and since the preparation of porcine LDH-III used in our experiments was homogeneous in gel diffusion tests, it is unlikely that our result is due to a contamination of LDH-III with an antigen unrelated to LDH. Hybrid-specific antibodies could not be detected by the quantitative precipitin test (see above) nor were they found by titration. Since antibodies are known to vary markedly in their capacity to agglutinate red blood cells, the size of the hybrid-specific antibody population may well be much smaller than the 10% suggested by passive hemagglutination inhibition. When individual antisera were tested, they reflected very closely in most cases the pattern shown in Fig. 6, regardless of whether primary or secondary antisera were used. Very rarely did the major antibody population of an antiserum seem to be better adapted to the hybrid enzyme than to the pure forms.

The Specificity of Secondary Stimulation.—Since by immunization with LDH-III the determinants of the A and B subunits are offered to the organism on the same molecule, it is of interest to ask whether in the terminal (productive) phase of the immune response the anti-A and anti-B antibodies are synthesized by independent production units, possibly different antibody-producing cells. Our experiments answered this question only partially but revealed an entirely unexpected aspect of the secondary response to LDH-III.

Rabbits were primed by injection of LDH-III in Freund's adjuvant. 4–5 wk later, they were boosted by successive intravenous injection of LDH-I and -V (or vice versa). Fig. 7 shows the experimental design and the two types of responses obtained in these experiments. Complete data are contained in Table II.

A strong booster response was induced in 19 of 20 animals by injection of LDH-V. 5 of 20 animals responded to injection of LDH-I with a booster response comparable in respect to anti-I titers to those obtained with the homologous antigen (cf. Fig. 3); the majority of the animals responded to that enzyme only slightly or not at all. In all cases, the injection of one of the pure types provoked, if successful, the formation of antibodies directed exclusively to that pure type. From the data of animals responding to both LDH-I and -V, it is obvious that the induction of a booster response to one of the pure types did not impair a subsequent booster response to the other.

A series of attempts was made to provoke regularly a secondary response to LDH-I in rabbits primed with the hybrid enzyme. A further intravenous injection of 5 mg of LDH-I had no effect, nor was intramuscular injection of 4.5 mg of LDH-I incorporated in Freund's complete adjuvant successful. Simultaneous secondary injection of LDH-I plus LDH-V was found to produce regularly a secondary response to LDH-V but not to LDH-I.

Since the LDH-III preparation used for immunization had been prepared in vitro by freezing and thawing of LDH-I and LDH-V in a certain milieu, LDH-I was submitted to the same treatment and recrystallized. The resulting

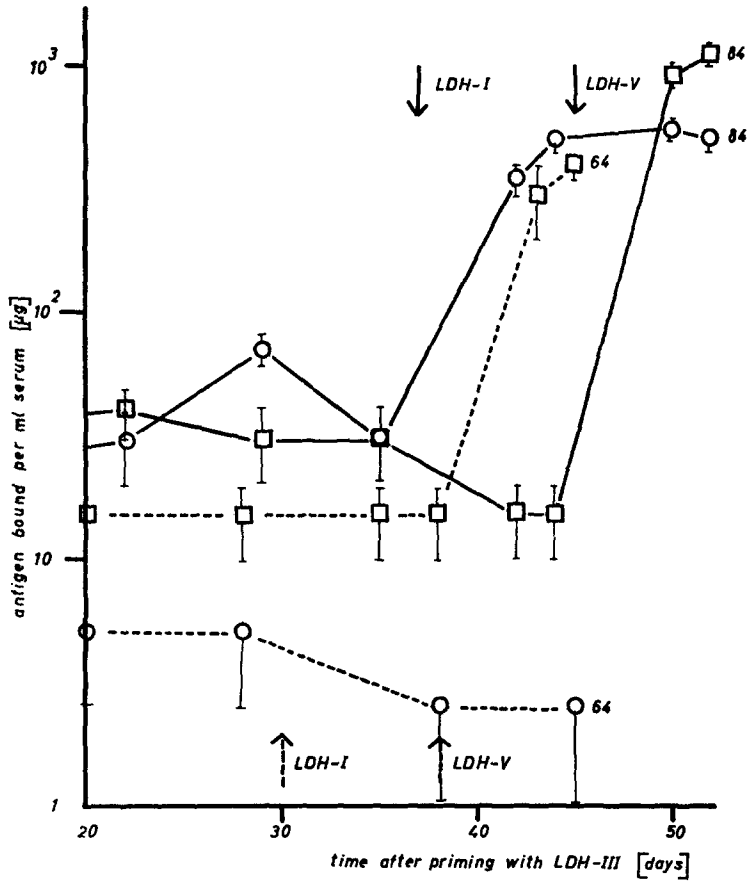

FIG. 7. The response of two representative rabbits (Nos. 6-4 and 8-4) primed with LDH-III to boosting injections of LDH-I and LDH-V. O—O, anti-I titers; □—□, anti-V titers. For further explanation see legend to Fig. 2.

enzyme preparation could not be distinguished from native LDH-I in gel diffusion tests, and was also not efficient in stimulating animals primed with LDH-III (1/7 responders).

At the time of secondary injection, the sera of all animals contained antibodies reacting with LDH-I. Passive hemagglutination experiments were carried out to test whether the specificity of these antibodies differed in responders and nonresponders to LDH-I. The two examples given in Fig. 8 are

TABLE II

Response of Rabbits Primed with LDH-III to Secondary Stimulation with LDH-I and LDH-V

Experiment No.	Rabbit No.	Antigen bound per ml serum, μg*					
		LDH-I	LDH-V	LDH-I	LDH-V	LDH-I	LDH-V
		Before secondary injection		7 days after secondary injection of LDH-I		7 days after subsequent injection of LDH-V	
1‡	6-3	5	5–10	60	10	40	100
	6-4	5	10–20	2.5	10–20	2.5	400
	6-5	5–10	10–20	2.5–5	10	2.5–5	400
	6-6	5–10	10–20	5	10–20	1–5	200–300
	6-7	1–2.5	10	20–40	5	10–20	10–20
2§	8-3	20–40	20–40	300–400	20–40	300–400	800
	8-4	20–40	20–40	500	10–20	500	1000–1200
3‖	1-35	40–60	20–40	150–200	20–40	150	1600–1800
	1-36	10	10–20	10–20	10–20	5	200–400
	1-37	10–20	20	20	20	5–10	800
	1-38	10–20	20–40	60–80	40	40–50	800
		Before secondary injection		7 days after secondary injection of LDH-V		7 days after subsequent injection of LDH-I	
1‡	6-8	10–20	20	10	800	5	600–800
	6-9	10–20	10–20	5	150	40	50–100
	7-0	5–10	10–20	5–10	300	20	200–300
	8-1	1	<5	1	50–100	2.5–5	20
	8-2	40	40–60	20	800	40–60	600
3‖	1-39	10	20–40	10	400–500	20–40	200–400
	1-40	40–60	40	20	1700	20–40	1200–1500
	1-41	20–40	20	20–40	500	20	400
	1-42	10–20	20	10–20	1000–1200	600	600–800

* Concentrations corresponding to definite excess of antibody (first number) and of antigen (second number) are given. Only one number appears if just a trace of antigen was detected in the supernatant at that concentration.

‡ Boosting injections on day 30 and day 38 after priming.

§ Boosting injections on day 37 and day 45 after priming.

‖ Boosting injections on day 32 and day 41 after priming. Rabbits 1-35–1-38 received on day 41 again LDH-I (5 mg) and were boosted with LDH-V 7 days later.

FIG. 8. Specificity of anti-I antibody in primary sera of animals primed with LDH-III and responding (No. 1-42) or not responding (No. 1-36) to secondary stimulation with LDH-I. Sera were adsorbed by LDH-V. Titers less than 3% (serum 1-36) or 8% (serum 1-42) correspond to serum dilutions <1:8. For further explanation see legend to Fig. 6.

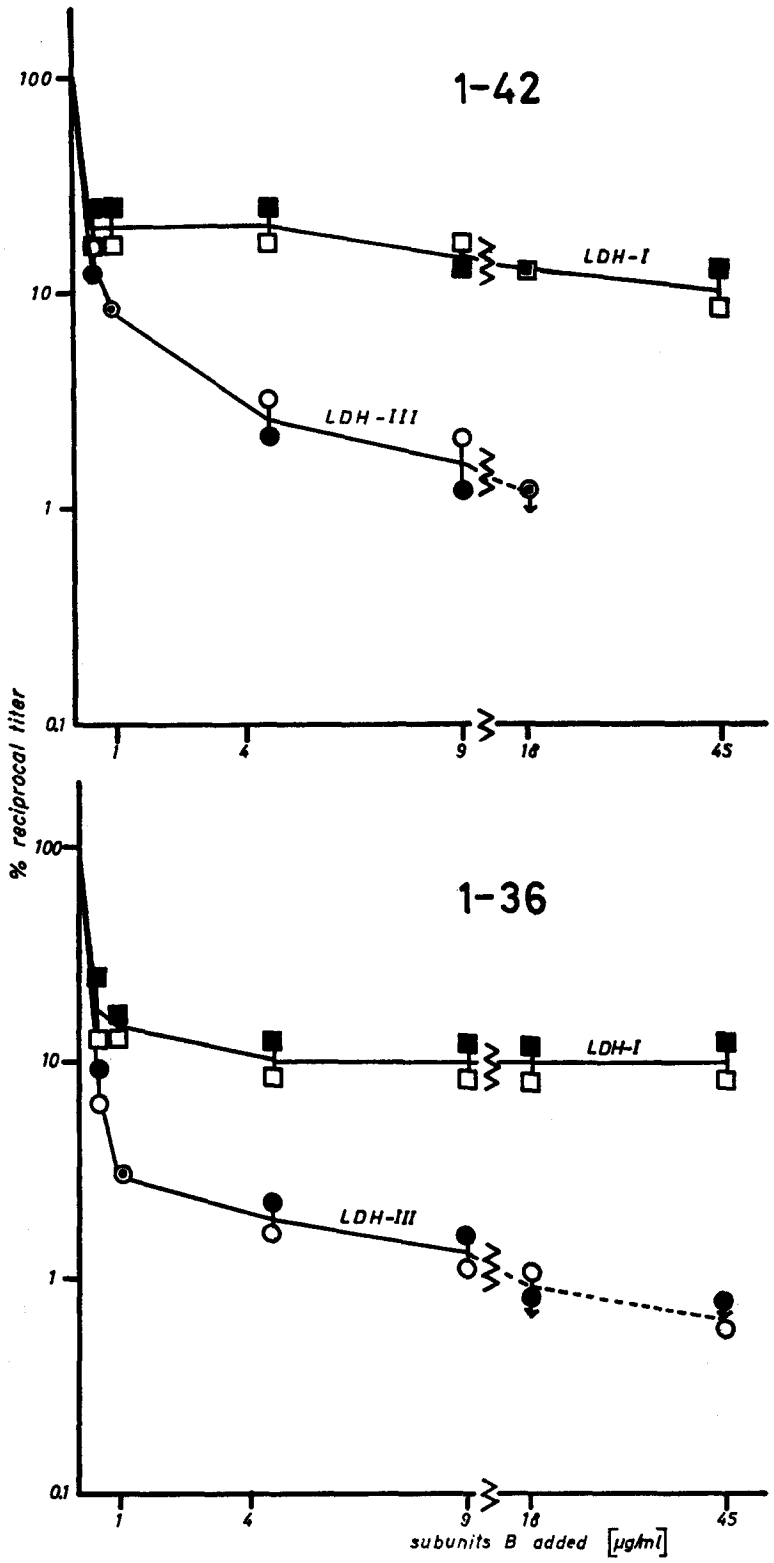
1-42
100
10
1
0.1
LDH-I
LDH-III
1
4
9
18
45
% reciprocal titer
1-36
100
10
1
0.1
LDH-I
LDH-III
1
4
9
18
45
subunits B added [µg/ml]

FIG. 8

representative of the results obtained. No significant difference was detected.

Specificity of Tolerance Induction. Induction of Hyporesponsiveness to LDH-III by Injection of LDH-V.—The following experiments were undertaken to study the influence of injection of LDH-I or LDH-V, respectively, into newborn rabbits on a subsequent immunization with the hybrid enzyme. Our interest was focused on two main questions. (*a*) Does injection of one of the pure types at birth depress the formation of antibodies to the respective moiety of the hybrid molecule only? (*b*) Is the predominant role of subunit A in the induction

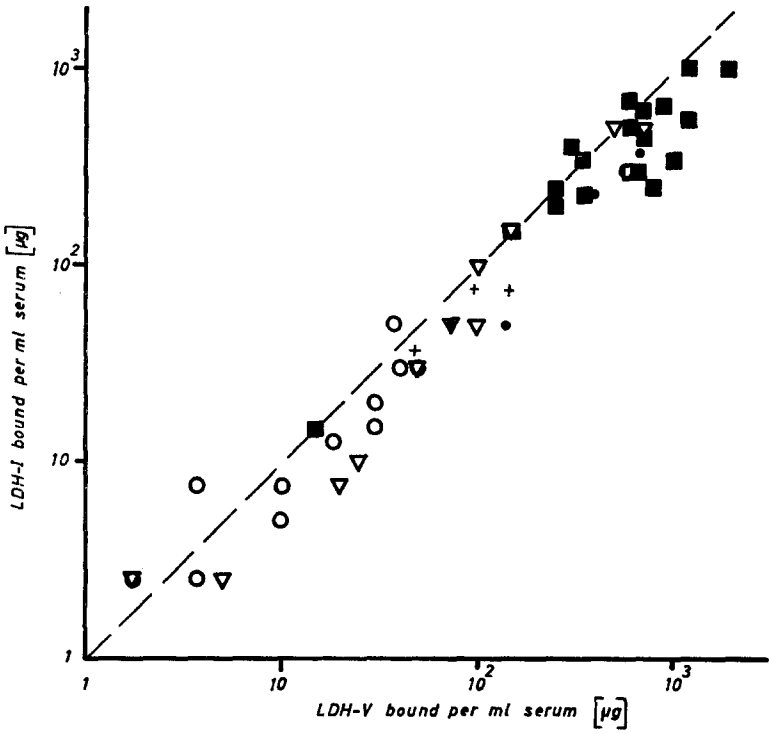

FIG. 9. Hyporesponsiveness to LDH-III induced by injection of LDH-V into the newborn. The responses of individual rabbits to LDH-III are represented in the diagram by single points indicating peak anti-I (ordinate) and anti-V titers (abscissa) after standard immunization. The dashed line is drawn to facilitate orientation to the ratio of the two titers. The diagram contains data of two independent experiments the results of which were statistically indistinguishable. Filled squares, control group; filled circles, 10 μg; crosses, 100 μg; open triangles, 1–2 mg; open circles, 10–40 mg of LDH-V at birth. Anti-I and anti-V titers of the control group are significantly higher than those of the 10–40 mg group ($P \ll 0.01$) and of the 1–2 mg group ($P < 0.05$). Anti-I titers of the 1–2 mg group are significantly higher than those of the 10–20 mg group ($P < 0.05$); the result was less clear for the anti-V titers ($0.1 > P > 0.05$).

of the secondary response to LDH-III reflected by a high capacity of LDH-V, as compared with LDH-I, to induce tolerance to LDH-III?

Rabbits were injected on the day of birth with varying doses of LDH-V. At the age of 12–14 wk, the animals were immunized with LDH-III by the standard immunization procedure. The results obtained are contained in Fig. 9 and they permit the following conclusions: (*a*) Complete unresponsiveness could not be achieved with any dose of LDH-V injected. (*b*) Injection of 10 mg and more of LDH-V at birth drastically depressed the immune response to LDH-III.

TABLE III

The Specificity of Hyporesponsiveness to LDH-III

Group of rabbits	Rabbit No.	Secondary response to human gamma globulin* (*μg antigen precipitated by 1 ml serum*‡)
Hyporesponsive§ to LDH-III	1-07	1200–1500
	1-08	1500–1800
	1-09	1800–2100
	1-21	1800–2100
	1-22	1500–1800
	1-25	900–1200
Controls	1-11	900–1200
	1-13	1800–2100
	1-20	1500–1800
	1-29	1500–1800

* Immunized according to the standard procedure, subsequent to immunization with LDH-III.

‡ Determined by adding increasing amounts of antigen to constant amounts of antiserum and testing the supernatants, after standard incubation, for the presence of antigen.

§ Equivalence points for the pure forms ranging from 2.5–40 μg.

Injection of 1 mg of LDH-V still led, in the majority of the animals, to a depressed response to the hybrid enzyme. Even as little as 100 μg of LDH-V given at birth seemed to exert some tolerogenic action, but the number of experimental animals is too low to permit a definite statement. (*c*) Injection of LDH-V at birth, if effective, depressed the complete response to LDH-III, i.e., the anti-V and the anti-I titers. In no case has a dissociation of the two antibody titers been found. It should be added that a number of precautions were taken upon immunization not to miss unexpected reactions of the animals injected at birth. Before the priming injection of LDH-III, the sera of these animals were titrated as routine with LDH-I and LDH-V; antibody activity could never be detected. It was also shown that animals injected at birth did not develop, after priming with LDH-III, any considerable secondary type

response. Animals with a depressed secondary response also showed a depressed primary reaction. The time course of the secondary reaction did not differ in these animals from the one found in the controls. The antibodies present in sera of animals showing a depressed response to LDH-III did not seem to differ in any respect from the antibodies in standard antisera to the hybrid enzyme. Again there was a majority of 7S IgG and a minority of 19S antibody as revealed by sucrose gradient centrifugation. Again they were composed of two antibody populations, one directed to LDH-I and the other to LDH-V: LDH-I

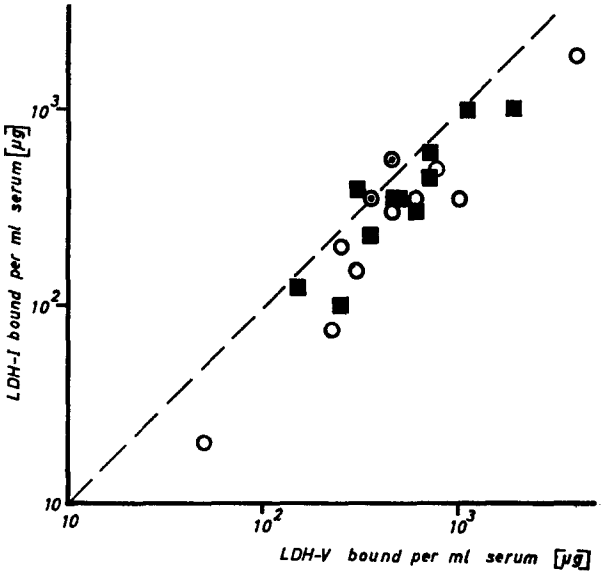

FIG. 10. Failure of LDH-I to induce hyporesponsiveness to LDH-III. Plot as in Fig. 9. Two independent, statistically indistinguishable experiments. Squares, control group; circles, 20–40 mg of LDH-I at birth. $P > 0.4$ for anti-I and anti-V titers.

and LDH-V showed reactions of nonidentity in double diffusion if compared by these antisera; and on titration the sera bound approximately twice as much LDH-III as either of the pure forms.

Depression of the response to LDH-III as induced by injection of LDH-V was not connected with a depression of the response to an unrelated antigen. Table III shows that tolerant and control animals responded strongly to immunization with human gamma globulin (gift of Dr. P. Grabar).

Failure of LDH-I to Induce Unresponsiveness to LDH-III or Acetylated LDH-I.—In contrast to LDH-V, LDH-I was found to be incapable of inducing hyporesponsiveness to LDH-III within the dose range tested. Injection of 20–40 mg of LDH-I in the neonatal period did not result in the animals in any

significant depression of the anti-I and anti-V titers upon immunization with LDH-III (Fig. 10).

We do not know whether LDH-I can exert any tolerogenic action at all. The direct test (namely injection of LDH-I into the newborn and subsequent immunization with the same enzyme) has not yet been carried out because of the irregular immune response to LDH-I in rabbits. It has been shown, however, that injection of 10–20 mg of LDH-I at birth did not affect the immune response of the animals towards acetylated LDH-I (Fig. 11). This enzyme, a potent immunogen, carries on its surface a few determinants created by the chemical

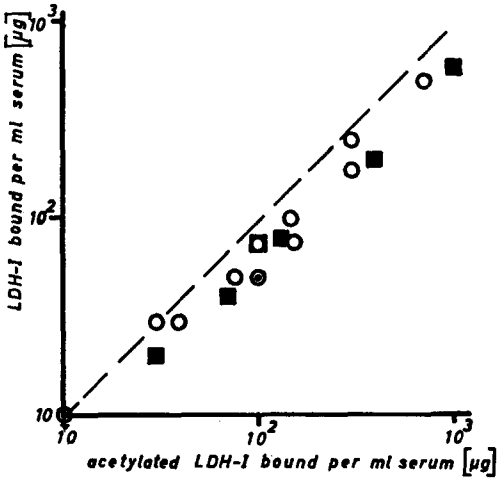

FIG. 11. Failure of LDH-I to induce hyporesponsiveness to acetylated LDH-I. Equivalence points for LDH-I are plotted versus those for acetylated LDH-I. Each point represents the response of an individual rabbit (cf. Fig. 9). Squares, control group; circles, 10-20 gm of LDH-I at birth. $P > 0.3$ for anti-I and anti-acetylated I titers.

treatment but also the complete set of antigenic determinants of the native enzyme (27).

There is, therefore, no evidence as yet for any tolerogenic action of LDH-I.

DISCUSSION

Characterization of the System.—Immunization of rabbits with porcine LDH-III leads to the production of anti-A and anti-B antibodies in comparable amounts. The ratio of anti-A to anti-B antibodies is remarkably constant. Thus, combining subunit A and B on one antigenic molecule results in a characteristic imprint on the immune response: We have recently found that priming with a mixture of LDH-I and LDH-V often leads to a dissociation of the anti-A and the anti-B titers. These experiments will be presented in another context,

since they have revealed further interesting features of the immune response to LDH.

It would seem, therefore, that the A and B subunits do not dissociate during the initiation of the immune response to LDH-III. This is also suggested by the stability of LDH-III under physiological conditions. We have kept the enzyme at a concentration of 50 μg/ml in physiological solution containing 40 mg BSA/ml for 5 days at 37°C without observing any considerable dissociation and reassociation of the subunits. In vitro hybridization of LDH subunits requires a rather drastic treatment of the enzyme(s) (26). Furthermore, ammonium sulphate, which is known to be a potent inhibitor of in vitro hybridization of LDH, was always present in the antigen-adjuvant mixtures used for immunization.

Fortunately, the combination of A and B subunits did not impose complexities and irregularities of antigenic structure on the hybrid molecule to any considerable extent. Virtually all precipitating antibody in anti-III sera can be precipitated by successive addition to the sera of LDH-I and LDH-V. There exists in the sera very little, if any, antibody reacting with both LDH-I and LDH-V. The majority of the antibodies present in the sera fit equally or almost equally well to one of the pure forms as to the hybrid enzyme, and only small amounts of antibodies specific for LDH-III are found. The system thus meets the immunochemical requirements of studies on the specificities involved in the induction of tolerance and of the secondary response.

Stimulation of Animals Primed with LDH-III by the Pure Forms.—In 1959, Dubert performed the following experiment (12): Rabbits were primed with a chemically modified human serum albumin and, after an interval of 31 days, boosted with the native protein. In accord with a previous observation by Dixon and Maurer (10), a booster response was obtained for both types of antibody inherent in the system, those directed to the native protein and others, specific for the chemically modified antigen. As Dubert pointed out, however, there was a slight cross-reaction of the latter antibodies with native human serum albumin.

We did not find a similar phenomenon in our system. Animals primed with LDH-III responded to injection of one of the pure types with a typical secondary response. However, the production of anti-A and anti-B antibodies can obviously be dissociated from one another. Since a secondary response to one of the pure types does not impair the subsequent initiation of a secondary response to the other, and since cell proliferation leading to production cells with a limited life span is thought to be an essential step in the secondary response (34), the simplest explanation for the observed effects is the assumption that the last step of the immune response to LDH-III involves cells specifically

producing anti-A or anti-B antibodies, but not both. However, as will be discussed below, this conclusion is not cogent, even if one assumes that the 1.5 mg of antigen injected led to an exhaustive stimulation. It should be kept in mind that upon stimulation of animals primed with LDH-III, only 5 of 20 developed the expected high antibody titers after injection of LDH-I, whereas 19 of 20 responded strongly to injection of LDH-V. In all animals, at the time of secondary stimulation, circulating antibodies to subunits A (LDH-V) and B (LDH-I) were present in the serum, sometimes in considerable amounts (Table II). The deficiency of LDH-I as a boosting agent can hardly be explained on the basis of serological specificity. Our immunochemical studies (see above) demonstrated a good fit to LDH-I of most of the anti-B antibodies provoked by sensitization with LDH-III. There was also no difference between LDH-I and LDH-V in this respect. Furthermore, primary antisera of animals which did not respond to secondary stimulation with LDH-I did not differ in specificity from primary antisera of responders (Fig. 8). The possibility that, because of a somewhat different serological specificity, LDH-I is a poor inducer of a secondary response in animals primed with LDH-III has been further excluded by experiments with a hybrid LDH molecule composed of porcine B and rabbit A subunits (33). This enzyme is very similar to porcine LDH-III in respect to the B-specific and the hybrid-specific determinants, but does not induce a secondary response in animals primed with the porcine hybrid enzyme.

It would thus seem that in the majority of cases, A subunits are necessarily involved in the process of induction of a secondary response to LDH-III. One might assume that priming with LDH-III leads to the development of a trapping mechanism (14, 15) ready to accept antigen for secondary stimulation. Subunits A would be required in the majority of our animals for a proper functioning of this trapping mechanism.

Further work is necessary to elucidate whether the finding of a minority of responders to secondary injection of LDH-I has a genetical background. It is of interest in this connection to note that standard immunization with LDH-I also led to very irregular results (Fig. 3). Some strains of laboratory animals are reportedly incapable of responding to certain antigens because of a genetically determined deficiency of initiating the immune response (35–38). The same would presumably be true in our system, since all rabbits are capable of forming anti-B antibodies in large amounts upon immunization with LDH-III.

Specificity of Tolerance Induction.—The main finding of our tolerance studies was that a tolerogenic injection of LDH-V depresses the complete antibody response to the hybrid enzyme, that is, both the anti-A and the anti-B titers; LDH-I does not exert a similar tolerogenic action. There are four possible ways of interpreting this result: (*a*) The LDH-V preparations used were contaminated

with hybrid enzymes. (*b*) The immune apparatus of the newborn rabbit cannot distinguish between LDH-I and LDH-V, but acquires this capacity during the first 3 months of life. (*c*) LDH-I and LDH-V, though not cross-reacting serologically, show the phenomenon of cross-tolerance. (*d*) Unresponsiveness to LDH-III is induced by blocking an early step of the immune response which is mediated by subunits A.

Contamination of our LDH-V preparations with hybrid enzymes is an unlikely cause of these results. If such a contamination existed, it must have been below 5×10^{-5}, as shown by passive hemagglutination inhibition. Since 1 mg of our preparation induced unresponsiveness in about 50% of the animals, one would have to assume that less than 0.05 μg of the hybrid enzyme exerted a strong tolerogenic action, which would be contrary to all previous experience (39).

Cross-tolerance of LDH-I and LDH-V, found either at birth only (possibility *b*) or as a general phenomenon (possibility *c*) in the rabbit, would seem unlikely from the data presented here since hyporesponsiveness to LDH-III could be induced by LDH-V, but not by LDH-I, in the dose range tested. The two enzymes seemed to differ strongly in this respect: Whereas 1 mg, and probably even 100 μg, of LDH-V was sufficient to induce hyporesponsiveness in a proportion of the animals, 40 mg of LDH-I had no effect at all. It could be argued, however, that LDH-I, though unable to induce tolerance, possesses a certain tolerogenic specificity, possibly identical with that of LDH-V. Further experimental work, which is included in a parallel paper (33), has shown that hyporesponsiveness to the hybrid molecule can be easily induced in adult rabbits, again by injection of LDH-V but not of LDH-I. The possibility that our results are attributable to delayed development of the ability to distinguish between subunits A and B is therefore excluded. Furthermore, cross-tolerance of LDH-I and LDH-V could be shown not to exist by direct testing. Thus there remains possibility (*d*), namely, the existence of a step requiring A subunits in the early phase of the immune response to LDH-III; this step could be blocked by a tolerogenic injection of LDH-V.

It is striking that the experiments on secondary stimulation and on tolerance induction independently lead to similar conclusions. Indeed, our data reveal a similarity of the processes of tolerance induction and of initiation of the immune response to LDH-III as to their specificity. This similarity is compatible with the view that the two processes are mediated by receptors which upon contact with antigen could either establish tolerance (probably the normal event in the case of LDH) or, in the presence of adjuvant, be transformed or multiplied in such a way that a further encounter of the organism with antigen leads to antibody formation. The specificity of the receptors in question cannot be defined from our data. However, we do not have any evidence for a participation of subunits B at that stage of the immune response in the majority of

our animals, and the results can be explained by attributing to the receptors involved just A-specificity. Whether this A-specificity is identical with the specificity of certain antibody combining sites is unknown. The results suggest only that the receptors we are dealing with probably are not conventional circulating antibody, since LDH-I was unable to stimulate the majority of our animals primed with the hybrid enzyme, though anti-B antibody was present in the sera at the time of secondary stimulation (see also Rajewsky and Rottländer (33)). On the other hand, it seems quite possible that the inductive phase of the immune response has its own specificity, which may or may not overlap with conventional serological specificity. This possibility has recently also been discussed in studies on the inheritance of the immune response (35–38). It should be noted in this connection that our system clearly shows a hapten-carrier effect, and the results may thus have a bearing on carrier effects in general. Together with the DNP-polylysine-BSA system (37), the LDH system represents an example of macromolecular haptens (the haptenic subunits B) being fixed to a carrier of similar size (the carrier subunits A). This aspect of the work is discussed in detail in a parallel paper (33). It may well be that hapten-carrier effects of the kind described here are a general phenomenon in that generally only certain parts of an antigen molecule possess carrier property and thus determine the specificity of that antigen during the early phase of the immune response. This could help to explain "breakdown" of tolerance and differences between tolerance specificity and the specificity of antibody.

The receptor concept clearly does not allow any conclusion to be drawn from our experiments on secondary stimulation with respect to a possible single commitment of the production cells. The question whether, in animals primed with the hybrid enzyme, the production cells produce either anti-A or anti-B antibodies or both has to be answered by direct testing. In a very recent study (40, 38) it was shown that in a similar system the antibody producing cells were committed to the production of one type of antibody only.

Finally, it should be pointed out that tolerance may well be inducible at several stages of the immune response. A very clearcut example of tolerance with respect to single antigenic determinants has been given by Schechter and coworkers (41), and various degrees of determinant specificity arise in numerous cases of experimentally induced unresponsiveness (see reference 42). Most striking, however, is the determinant specificity of natural tolerance. The factors that determine whether tolerance is induced at an early or (in addition?) at a late stage of the immune response are not yet clearly defined.

SUMMARY

Upon immunization with LDH-III (subunit composition AABB) rabbits produce anti-A and anti-B antibodies in comparable amounts. These antibodies fit equally well to the hybrid enzyme and to LDH-V (AAAA) or LDH-I

(BBBB) respectively, as tested by passive hemagglutination inhibition. No antibodies reacting with both LDH-I and LDH-V were detected. A minority of hybrid-specific antibodies was, however, present in the sera.

Animals primed with LDH-III respond regularly to a boosting injection of LDH-V with the production of large amounts of anti-A (but not anti-B) antibodies. A similar injection of LDH-I stimulates (if it has any effect at all) the production of anti-B antibodies only. Stimulation with one of the pure types does not impair a subsequent response to the other.

The majority of the animals primed with LDH-III responded not at all or weakly to a boosting injection of LDH-I, though antibodies to LDH-I were present in the sera at the time of stimulation. This effect can hardly be explained on the basis of serological sepcificity.

Hyporesponsiveness to LDH-III can be induced by injection of LDH-V into the newborn. Both anti-A and anti-B titers are equally depressed. Within the dose range tested, LDH-I does not exert any tolerogenic action with respect to LDH-III.

The carrier property of subunit A is evident in the induction of both immunity and tolerance to LDH-III. The early phase of the immune response to the hybrid enzyme may be carrier-specific, and receptors for the haptenic subunit B may not exist at that stage.

We wish to thank Professor N. K. Jerne for stimulating discussions, and Prof. J. Harte for suggesting to us the use of rank correlation tests. We are, furthermore, most grateful to Drs. H. Kneser, H. S. Micklem, I. Müller, and Mrs. U. Kläsener for advice and help when the work was written up. Finally, thanks are due to Mr. G. von Witzleben for technical assistance.

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