AN APPROACH TO THE QUANTITATION OF IMMUNOGENIC ANTIGEN*

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In vivo tracer studies of labeled antigens employing either quantitative tissue extraction techniques or radioautography are extremely sensitive in detecting antigen molecules or their degradative products. However, these procedures are unable to differentiate between antigenic material involved in stimulating antibody formation (immunogen or immunogenic antigen) and antigenic material which is being sequestered and/or catabolized as foreign substance without relation to the antibody response. The difference between immunogenic and nonimmunogenic forms of a given antigen or its fragments might be determined by its physical or chemical modification within the host, or by its anatomical localization. In addition, the capacity of the host to respond limits the total possible immunogen, i.e., every host has a limited number of immunologically responsive cells and only antigen involved in stimulating these cells could be immunogenic while additional antigen even in identical form would be wasted and, therefore, nonimmunogenic. The inability of antigen tracer techniques to differentiate between immunogenic and nonimmunogenic antigen is a serious limitation for if, as seems likely, the immunogenic fraction derived from an administered antigenic dose is extremely small and the traceable fraction significantly larger, most of the material traced would be of little immunologic consequence.

The present experiments were designed to obtain a quantitative estimate of the total immunogenic antigen involved in the stimulation of a primary antibody response. The experimental design was based on the results of numerous studies of the inhibition of antibody synthesis by passively administered antibody. It appears clear that this inhibition is caused by the neutralization of the antigenic stimulus by the passive antibody (reviewed by Uhr and Möller) (1) and, further, that the effective block is at the level of individual antigenic

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determinants (2, 3). If, as seems likely, this inhibition involves combination of passively administered antibody with immunogenic antigenic determinants, then the antibody so combined should be able to be differentiated from free uncombined antibody and the amount of this combined antibody should be proportional to the amount of immunogenic antigen, plus any residual non-immunogenic antigen available to react with the antibody. Thus, such a measure could establish an upper limit for the amount of immunogen present in the responding animal. To obtain such a measure, isotope-labeled, passively administered antibody has been used to inhibit the primary antibody response of rabbits to keyhole limpet hemocyanin (KLH) and the portion of the labeled antibody removed from the circulation and/or retained in the tissues during the process was determined.

Materials and Methods

Albino male rabbits weighing 5–6 lb. were used as experimental subjects and were maintained on iodide-containing drinking water. Associated KLH purified by ultracentrifugation (4) and bovine serum albumin (BSA) were used as antigens. Three pools of hyperimmune sera to KLH and two pools of hyperimmune sera to BSA from rabbits repeatedly immunized by a variety of routes over a period of 3 or more months were the sources of antibodies.

Anti-KLH determinations were made with a modified quantitative precipitin technique using dissociated KLH as previously described (5). The results are expressed as micrograms of dissociated KLH nitrogen precipitated by 1 ml of antiserum at the point where 80% of the KLH added is precipitated (P^{80}). 1 anti-KLH P^{80} unit consists of about 11 μg antibody protein (2).

Iodination of IgG and $F(ab')_2$ with ^{131}I or ^{125}I was performed according to the method described by McConahey and Dixon (6). Less than 2% of the radioactivity of all preparations were nonprotein bound as determined by trichloroacetic acid (TCA) precipitation.

Isolation of Immunoglobulin G (IgG).—The crude globulin fraction from pooled rabbit hyperimmune serum to KLH, obtained by precipitation at 50% saturation of ammonium sulfate, was dialyzed against 0.01 M sodium phosphate buffer, pH 7.6, and passed through a column of DEAE-cellulose equilibrated with the same buffer. The protein peak eluted with the same buffer was concentrated by means of negative pressure dialysis to a concentration of about 20 mg/ml and dialyzed against phosphate buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2). 1 ml aliquots were stored at −70°C.

The globulin preparation formed a single precipitin line with slow electrophoretic mobility when analyzed by immunoelectrophoresis with a goat anti–whole rabbit serum. Ultracentrifugal analysis of the IgG preparation revealed a single symmetrical peak with a sedimentation coefficient of approximately 6.6S. Anti-KLH antibody titer of purified IgG from two different pools ranged from 57 to 75 P^{80}/mg protein, i.e., 40–60% of the IgG was specific anti-KLH as determined by a quantitative precipitin analysis.

The same method was employed to isolate IgG from pooled rabbit hyperimmune serum to BSA. Anti-BSA antibody titer of purified IgG varied from 20 to 23 P^{80}/mg protein.

Isolation of $F(ab')_2$ Fragment.—Pepsin digestion of IgG was performed as described by Nisonoff et al. (7). 300 mg IgG were dialyzed against 0.1 M sodium acetate buffer, pH 4.0, at a concentration of 20 mg protein/ml. 2 mg of twice crystallized pepsin (Worthington Biochemical Co., Freehold, N. J.) were added per 100 mg IgG. The digestion continued for 18 hr at 37°C and was stopped by raising the pH of the reaction mixture to 8.0 with 1 N sodium hydroxide. The digestion product was further separated by gel filtration on Sephadex G-150

(Pharmacia, Uppsala, Sweden). The protein peak corresponding to $F(ab')_2$ was concentrated by means of negative pressure dialysis to a concentration of about 20 mg/ml and dialyzed against phosphate-buffered saline. Ultracentrifugal analysis of $F(ab')_2$ revealed a single symmetrical peak with a sedimentation coefficient of about 5S. In double diffusion studies, $F(ab')_2$ formed a single precipitin line with a goat antiserum to rabbit Fab fragment (kindly provided by Dr. H. M. Grey), but did not react with a sheep antiserum to rabbit Fc fragment. Anti-KLH antibody titer of purified $F(ab')_2$ ranged from 48 to 73 P^{80}/mg protein.

Determination of IgG Elimination.—In the single labeled antibody experiments, rabbits were injected intravenously with 1.0 ml of labeled IgG prepared from hyperimmune anti-KLH sera. The absolute number of counts injected per rabbit was determined by counting the individual syringes before and after injection. At various intervals thereafter, 3 ml aliquots of blood were collected from the ear vein in tubes containing one drop of dipotassium ethylenediamine tetraacetate. The protein-bound radioactivity present in 0.5 ml of plasma was determined in duplicate after TCA precipitation. After correction for decay, the counts were converted to per cent of injected protein remaining in the plasma using an arbitrary plasma volume of 39.1 ml/kg body weight.

Paired label experiments were carried out using a mixture of ^{131}I-labeled anti-KLH IgG and ^{125}I-labeled anti-BSA IgG. In order to minimize the possibility of injecting aggregates, the mixture was ultracentrifuged at 105,000 *g* for 90 min in a Spinco model L preparative ultracentrifuge. The upper two-thirds of the tubes were carefully decanted and injected within the next hour.

Per cents of injected ^{131}I anti-KLH and ^{125}I anti-BSA remaining in the circulation of each rabbit were determined as described above. From these individual per cents, group averages were calculated. The average per cents observed in the control rabbits receiving no antigen were then used as reference figures. Since there is no reason other than random variations in blood volumes, catabolic rates, etc., for observed differences between the per cent of ^{125}I anti-BSA remaining in KLH injected rabbits and rabbits receiving no KLH, the ^{125}I anti-BSA per cent in the KLH injected rabbits was adjusted to the reference value determined in the rabbits receiving no KLH. It was assumed that the same random variations in each rabbit would affect similarly the serum ^{131}I anti-KLH values, and they were adjusted by the same factor necessary to adjust the ^{125}I anti-BSA per cents. The difference between the per cent ^{131}I anti-KLH in the rabbits receiving no antigen and the corrected per cent ^{131}I anti-KLH in the rabbits receiving KLH was considered the per cent utilization of the passively administered anti-KLH as a result of reaction with persisting KLH.

Determination of $F(ab')_2$ Retention.—Rabbits were injected with 1.0 ml ^{131}I-labeled anti-KLH $F(ab')_2$. The total amount of ^{131}I retained by the animal was followed by external whole body counting using a specially designed cylindrical plastic phosphor whole body detector (manufactured by Efco Systems, Canoga Park, Calif.). In order to eliminate errors caused by ^{131}I in bladder urine, rabbits not urinating spontaneously before being counted or presenting a high level of radioactivity were catheterized. The counts were converted to per cent injected radioactivity after correction for decay.

EXPERIMENTAL PROCEDURES

Three types of experiment have been done. In the first, ^{131}I-labeled rabbit IgG consisting of approximately 50% anti-KLH was injected in amounts of from 2 to 20 P^{80} units into three groups of rabbits: one group had been immunized intravenously with 2 mg native KLH 1 day before, the second had been immunized subcutaneously with 2 μg native KLH in incomplete Freund's adjuvant (ICFA) just prior to antibody injection, and the third had not received KLH. In all rabbits, the levels of protein-

bound ^{131}I in the plasma were determined from 1 to 5 days after its injection. The difference between the amounts of labeled IgG circulating in nonimmunized rabbits and in immunized rabbits was considered to be the result of combinations of anti-KLH with persisting KLH.

The second type of experiment employed ^{131}I-labeled $F(ab')_2$ fragments prepared from the anti-KLH IgG. 9 or 18 P^{80} units of $F(ab')_2$ fragments were given to nonimmunized rabbits or to rabbits 1 day after the injection of 1 mg KLH intravenously. Since $F(ab')_2$ fragments lacking the Fc piece are rapidly removed from the circulation in the absence of antigen, 95% in 1 day, and excreted in the urine (8), the residual ^{131}I in these rabbits was determined by whole body counting daily for 5 days.

TABLE I

Antigen-Dependent Elimination of Passively Administered ^{131}I-Anti-KLH IgG from the Circulation and Associated Immunosuppression

Passive antibody	Exp. No.	No. of rabbits	Specific elimination*					Inhibition of day 20 control antibody response
			Day 1	Day 2	Day 3	Day 4	Day 5	
P^{80} units			%	%	%	%	%	%
			2 mg KLH i.v.					
2	I	5	4.2	1.3		0.1		29
3	IV	4	5.7	3.8	2.9	2.5	2.2	36
10	I	5	3.6	1.2		0.03		83
10	II	6	1.7		0.1		0.9	95
10	III	6	0.8		0.5		1.0	91
20	IV	4	1.9	1.4	0.2	0.7	0.2	84
			2 μg KLH in ICFA					
10	II	7	0.7		2.8		2.0	73
10	III	6	0		2.2		2.4	46

* Figures are the differences between the per cents of injected ^{131}I-anti-KLH IgG present in circulation of nonimmunized and immunized rabbits.

The rapid renal elimination of immunologically uninvolved $F(ab')_2$ removed most of the undesirable background counts which persisted in the first type of experiment. The increased retention of $F(ab')_2$ fragments in immunized rabbits over the nonimmunized controls was considered the result of combination with persisting KLH.

The third type of experiment employed the paired label technique. Doses of 2, 10, or 50 P^{80} units of labeled anti-KLH IgG and comparable amounts of labeled anti-BSA IgG were both injected into three groups of rabbits: one group had been immunized with either 2 mg or 50 mg KLH intravenously 1 day before, the second group had been immunized with 2 μg KLH in ICFA immediately before, and the third group was not immunized. In two such experiments, the anti-KLH was labeled with ^{131}I and the anti-BSA with ^{125}I and in one experiment, the labels were reversed. In all experiments, the amount of protein-bound ^{131}I and ^{125}I in the plasma was determined for 6 days after injection.

RESULTS

The results of the studies in which immunosuppressive doses of ^{131}I-labeled anti-KLH IgG were intravenously given to nonimmunized rabbits, to rabbits 1 day after the injection of 2 mg KLH intravenously, or to rabbits immediately after subcutaneous injection of 2 μg KLH in ICFA are shown in Table I. The figures are the differences between the amounts of circulating anti-KLH IgG in nonimmunized and immunized rabbits. The average per cent of the protein-bound radioactivity present in the plasma was constantly lower in the rabbits injected with KLH than in the nonimmunized rabbits. However, these differences were rather small. With the strongly immunosuppressive dose of 10 P^{80} units, there were, during the first day, only 0.8–3.6% of the original dose specifically removed from the circulation of the immunized rabbits. As would

TABLE II

Antigen-Dependent Whole Body Retention of ^{131}I-Anti-KLH F(ab')$_2$ Passively Administered 1 Day after Injection of 1 mg KLH Intravenously and Associated Immunosuppression

Passive antibody	No. of rabbits	Per cent antigen-dependent retention*			Per cent inhibition of day 20 control response
		Day 1	Day 2	Day 3	
P^{80} units					
9	8	4.1	2.1	0.9	64.2
9	7	4.6	0.3	0.3	52.2
18	7	2.9	1.5	0.6	77.3

* Figures are the differences between whole body retention of anti-KLH F(ab')$_2$ in immunized and nonimmunized rabbits.

be expected, there tended to be a larger per cent specific utilization with the smaller doses of passive antibody, 2 and 3 P^{80} units, than with the larger doses, although this was not consistent. During the 4 following days, a detectable but smaller difference was still apparent. In the rabbits injected subcutaneously with 2 μg KLH in ICFA, there was little or no specific removal of ^{131}I anti-KLH IgG from the circulation during the first 24 hr. However, 2.0–2.8% of 10 P^{80} units were specifically removed from the circulation of the immunized rabbits during the following days.

The results of the experiments utilizing passively administered F(ab')$_2$ fragments of anti-KLH to cause immunosuppression are indicated in Table II. Again, the values are the differences between retention in immunized and nonimmunized rabbits. The whole body radioactivity of rabbits injected intravenously with 1 mg of KLH and ^{131}I-labeled anti-KLH F(ab')$_2$ 24 hr later was slightly higher than that of rabbits injected with anti-KLH F(ab')$_2$ alone. The amount of antigen-dependent retention after 1 day was somewhat greater

than 4% when 9 P^{80} units of passive antibody were injected and 2.9% after 18 P^{80} units. As with intact anti-KLH, the antigen-dependent effect decreased over the next several days. As might be expected with an antibody preparation so rapidly excreted in the urine as is $F(ab')_2$, the immunosuppressive effect was less than that seen after injection of intact anti-KLH.

The results of the experiments, in which a mixture of labeled anti-KLH IgG and labeled anti-BSA IgG were given intravenously to rabbits immunized with KLH or to nonimmunized rabbits, are shown in Table III. The figures are the

TABLE III

Antigen-Dependent Elimination of Passively Administered Anti-KLH IgG from the Circulation as Determined by Paired Label Technique and Associated Immunosuppression

Passive antibody	Exp. No.*	No. of rabbits	Utilization‡			Inhibition of day 20 control response
			Day 1	Day 2	Day 4	
P^{80} units			%	%	%	%
			2 mg KLH i.v.			
2	VII	8	1.3	1.3	1.1	0
10	V	7	1.1	1.7	1.7	75
10	V	6	2.9	1.0	1.0	62
10	VI	8	0.4	0.7	0.5	69
10	VII	5	1.0	0.9	0.7	83
50	VII	7	−0.3§	−0.2§	−0.1§	97
			50 mg KLH i.v.			
10	V	3	20.2	15.7	12.2	
			2 μg KLH in ICFA			
10	V	5	2.6	0.8	1.1	73

* In Experiments V and VII, anti-KLH was labeled with ^{131}I and anti-BSA with ^{125}I, and in Experiment VI the labels were reversed.

‡ Figures are the differences between the per cents of injected anti-KLH IgG present in the circulation of nonimmunized and immunized rabbits.

§ Negative values indicating an insignificant retention with 50 P^{80} units in immunized rabbits.

differences between levels of circulating anti-KLH in nonimmunized and immunized rabbits. Use of the paired label technique eliminated the complication of any random variation of normal IgG metabolism among the different groups of rabbits. When anti-KLH IgG from three different pools was given intravenously to rabbits 1 day after intravenous injection of 2 mg KLH, from 0.4 to 2.9% more of the 10 P^{80} units were removed from the circulation during the 1st day than were removed in nonimmunized rabbits. When 50 P^{80} units were given, no utilization in the immunized rabbits was seen. Similar per cents of antigen-dependent elimination were obtained after intravenous administration of 10 P^{80} units to rabbits injected with 2 μg KLH in ICFA. However, when

the KLH intravenous challenge was raised 25 times, to 50 mg, the per cent utilization of 10 P^{80} units was increased approximately 15 times suggesting a direct relationship between amount of injected KLH and anti-KLH utilization.

DISCUSSION

The amount of passively administered antibody removed from the circulation and/or rapidly catabolized during the suppression of the primary response to KLH is extremely small. This is no doubt due in large part to the rapid catabolism and/or elimination of KLH by normal, nonimmune rabbits. Within 1 day, far less than 1% of intravenously injected KLH remains in the circulation and elimination of most of the antigen from the tissues is also rapid (5). Thus, in comparison with many other antigens, there is relatively little KLH persisting 1 day after its injection when inhibiting antibody is administered, and hopefully a sizeable part of the retained KLH is immunogenic. By two different techniques, the amount of anti-KLH utilized in the course of immunosuppression was shown to be between 0.7 and 2.9% of 100 μg anti-KLH IgG injected 1 day after an intravenous injection of 2 mg KLH in 3 kg rabbits. Measurement of the retention of anti-KLH $F(ab')_2$ molecules in the whole body of rabbits with and without KLH indicated that from 4.1 to 4.6% of 63 μg were retained specifically in the presence of antigen and that 2.9% of 126 μg were retained under similar conditions. These small differences in the metabolism of antibody in the presence or absence of antigen and in the antigen-related handling of control IgG and specific antibody, or its fragments, are probably significant in view of their consistency, especially in the paired label experiments.

If we assume that at least part of the antibody specifically removed from the circulation or retained in the tissues is responsible for the observed immunosuppression, it is possible to translate the antibody utilized into an amount of antigen with which it might react and neutralize. It should be remembered, however, that not all of the antigen reacting with the administered antibody need be immunogenic since any persisting antigen, in a site available to the injected antibody, could combine with it regardless of whether it was stimulating antibody synthesis or not. Thus, any estimate of immunogen based on the present studies might be falsely high. Earlier, it was determined that anti-KLH mixed with the KLH and incorporated in incomplete Freund's adjuvant could suppress the expected antibody response. In this situation, 12 μg anti-KLH mixed with 2 μg KLH inhibited the antibody response approximately 80% over the first month (2). Making the conservative estimate that all KLH in the adjuvant mixture was immunogenic, the anti-KLH/KLH weight ratio needed for effective suppression was at least six. If this ratio is applied to the amounts of intravenously administered anti-KLH apparently utilized in immunosuppression (1–3 μg), it would indicate that about 0.2 μg–0.5 μg of KLH

could have been neutralized. In a 3 kg rabbit, the antibody response to the injection of 2 mg KLH, i.e. a maximum of 0.2–0.5 μg immunogen, averages about 9 P^{80} units/ml of serum or about 30 mg anti-KLH in the entire rabbit, a level which is maintained for several weeks (5). While the anti-KLH/KLH ratio needed for neutralization in the presence and absence of adjuvant could differ, the fact that the blocking apparently occurs at the level of individual antigenic determinants would suggest that a reasonably constant ratio might prevail.

That at most only 0.2–0.5 μg of immunogenic antigen might result from an intravenous injection of 2 mg of KLH is consistent with the observed antigenicity of KLH administered in adjuvant. 2 μg of KLH incorporated in ICFA elicits an antibody response comparable in size to that which follows the intravenous injection of 2 mg KLH. Since not all of the KLH in the emulsion would be released in the first several weeks after injection and since not all that was released would necessarily be immunogenic, the immunogen provided by 2 μg of KLH in ICFA could well be near the 0.2–0.5 μg range, thereby accounting for the comparable antibody responses. Significantly, the amount of labeled, intravenously administered anti-KLH specifically removed from the circulation in rabbits injected with 2 μg KLH in ICFA was within the same range as that removed in rabbits injected with 2 mg intravenously.

Since interpretation of the present observations demands a number of assumptions which cannot be verified and no doubt introduce errors, the calculations can, at best, be considered approximations. However, in each instance, we have attempted to use conservative estimates and assumptions so that our calculations of immunogenic antigen are more likely high than low. If this is true, the amount of immunogen involved in stimulating significant antibody responses may indeed be so small that they would be difficult to detect even with our most sensitive tracer techniques. Also, if in vivo tracer studies detect significantly larger proportions of injected antigen than indicated as immunogen in these experiments, it is possible that much of what is being traced is not immunogenic. Refinement and application of the present technique to other host-antigen systems should provide valuable guide lines for future attempts at antigen tracing.

SUMMARY

Using passively administered isotope-labeled anti-KLH to suppress the antibody response of rabbits to KLH, we have attempted to estimate the amount of antigen actually involved in stimulating antibody formation. Single and paired label tracer studies of passively administered anti-KLH IgG indicated that from 0.7 to 2.9 μg were utilized or involved by the antigen in the course of a 90% suppression of the response to 2 mg KLH. Tracer studies of labeled anti-KLH $F(ab')_2$ fragments revealed the retention of from 2 to 3 μg of these fragments in the entire rabbit during a 60% suppression of the re-

sponse to 1 mg KLH. Based on previously determined ratios of mixtures of KLH and suppressive amounts of anti-KLH in adjuvant, the antibody utilization data were converted to the probable amount of antigen or immunogen involved. It appears that after an injection of 2 mg of KLH approximately 0.2–0.5 μg of antigen persisted and reacted with antibody given 24 hr later. Since all of this persisting, reactive antigen may not be immunogenic, the above estimate of immunogen is probably high, but may serve to establish upper limits for the amounts of immunogen involved in stimulating antibody formation and provide a meaningful frame of reference for antigen tracer studies.

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