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Carrier-Determined Suppression of Anti-fluorescein Antibody in the Rabbit (39428)

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Immunologic tolerance to simple hapten bound to nonimmunogenic carriers has gained wide acceptance to study the mechanism of tolerance (1-6). Of particular interest is autologous IgG as a tolerance-inducing carrier because it is a physiologic protein naturally tolerated by the host (5). Although this phenomenon has been observed in several strains of mice (7), it is not known whether this is a general property of autologous IgG. For this reason, we have examined the tolerogenic properties of autologous IgG in a different system, the anti-fluorescein response of rabbits (8). This particular system was chosen for two reasons. Firstly, the rabbit anti-fluorescein (anti-Fl) response to an immunogenic hapten carrier conjugate (Fl-KLH) (Fl-keyhole limpet haemocyanin) has already been extensively characterized in this laboratory (8). Secondly, if one can induced tolerance to a fluorescent hapten it will offer distinct advantages to follow the fate of the tolerogen in vivo.

Materials and methods. Preparation of hapten carrier conjugates. Keyhole limpet haemocyanin (KLH) and purified isologous rabbit gamma globulin (RGG) were obtained commercially from Schwartz-Mann. Autologous RGG was purified from the serum of individual rabbits by starch block electrophoresis. Fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, Mo.) was conjugated in different molar ratios to KLH, RGG (homologous), and RGG (autologous) by previously described methods (8).

Immunizations. Randomly bred New Zealand white rabbits (Gloucester Rabbitry) were used throughout these studies. The rabbits were injected subcutaneously (in the rear footpads) with 3-5 mg of hapten carrier conjugate in complete Freund's adjuvant (CFA), or intravenously (in the marginal ear vein) with 30-50 mg of the hapten carrier conjugate in phosphate-buffered saline (PBS). For iv injections the molar ratio of hapten to carrier was 8-10:1 based on molecular weights of 900,000 for KLH and 150,000 for RGG. For sc injections the molar ratios were 85:1 for Fl-KLH and 8:1 for Fl-RGG. In some experiments iv and sc injections were performed simultaneously.

Anti-Fl assays. In these studies, the relative levels of anti-Fl responses to different hapten carrier conjugates and different methods of immunization were compared in terms of the maximum concentration of anti-Fl antibody reached in the serum following each injection.

The rabbits were bled weekly (35-40 ml) and screened for anti-Fl activity by qualitative precipitin tests with Fl-bovine serum albumin (BSA). (8) The RGG fraction in each serum sample was precipitated by $(NH_4)_2SO_4$ (30% w/w), dissolved in PBS (25 ml) and dialyzed against PBS to remove any traces of $(NH_2)_2SO_4$. The RGG solutions were then diluted to 0.5-1.0 mg/ml and assayed by fluorescence titrations (9) to determine the anti-Fl concentration. The actual concentration of anti-Fl in the original serum was calculated from this data. Using this technique, serum antifluorescein levels as low as 0.1 mg/ml could be detected.

Anti-KLH assay. The relative concentrations of anti-KLH antibody were determined by a quantitative precipitin test in which the precipitated antigen-antibody complex was assayed spectrophotometrically. The extinction coefficient ($E \pm \frac{cm/ml}{mg}$) of IgG and KLH were assumed to be 1.5 (8) and 1.7 (10), respectively. The weight of anti-KLH antibody in the precipitate was then used to calculated the concentration (in milligram per milliliter) of anti-KLH anti-

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body in the original serum.

Results. Induction of immunological tolerance to Fl by Fl-RGG (autologous). Previously, it has been shown that immunization with the antigen Fl-KLH (sc) induces an anti-Fl response in rabbits. The maximum reached within 2 weeks was in the range of 1–2 mg/ml (8) and is maintained for 2 to 3 weeks, then declines after 6 weeks. At this time the serum anti-Fl concentration is less than 0.1 mg/ml, the limit of detection in our assay. The effect of treatment with Fl-RGG (autologous) on the anti-Fl response was studied. The results are shown in Table I.

Animals (Group I) injected with Fl-RGG (autologous) iv had no detectable antibody to Fl in their serum either prior to or after a second iv injection of Fl_8-RGG (autologous) together with a subcutaneous injection of Fl_{85}-KLH in CFA. In contrast, animals similarly treated with either Fl-RGG (homologous) (Group II) or Fl-KLH (Group III) produce an anti-Fl response.

To determine whether pretreatment with the autologous hapten-carrier conjugate was necessary for the induction of tolerance, three untreated rabbits were injected, first with Fl_8-RGG (autologous) iv, and then, immediately thereafter, with Fl_{85}-KLH sc. No anti-Fl antibody was detected in any of the rabbits during the next 7 weeks.

Suppression of the secondary response to Fl by Fl-RGG (autologous). The results are shown in Table II. Six rabbits (Group IV) were immunized sc with Fl_{85}-KLH and bled weekly until the concentration of anti-Fl in the serum was less than 0.1 mg/ml. During this period, (6 to 8 weeks) the concentration of anti-Fl in the sera reached maximum levels of 1–2 mg/ml.

Each of the rabbits was then injected simultaneously with Fl_8-RGG (autologous) and Fl_{85}-KLH sc. Five of the six rabbits produced no detectable anti-Fl antibody during the next 6 weeks, and, in the sixth rabbit, the response was suppressed (0.3 mg/ml). In a control experiment, five rabbits similarly treated with Fl_{85}-KLH (Group V) showed a normal anti-Fl response (1–2 mg/ml) to both primary and secondary injections.

Seven weeks after the tolerizing injection, each of the tolerized rabbits were challenged with sc injection of Fl_{85}-KLH. In each case, the normal anti-Fl response was restored.

Table I and II show that the suppression of the fluorescein response is hapten specific since the immune response to the carrier (KLH) is the same in control rabbits as well as in rabbits made tolerant to fluorescein.

Immunogenicity of all Fl Carrier Conjugates. The results show (Table III), as expected, that Fl-KLH is more immunogenic than Fl-homologous RGG and that the subcutaneous route of administration is more effective than the intravenous route in inducing antibody formation. In contrast, Fl-

TABLE I. SUPPRESSION OF THE PRIMARY RESPONSE TO Fl BY Fl-RGG (AUTOLOGOUS)

Group	No. of rabbits	Experimental schedule (week)	Conjugate	Route of administration	Average maximum anti-Fl concentration in serum (mg/ml)[a]	Average maximum anti-KLH concentration in serum (mg/ml)
I	5	0	Fl-RGG (Autologous)	IV	<0.1[b]	–
		8	Fl-RGG (Autologous)	IV	<0.1	0.73 (0.03)
			Fl-KLH	SC		
II	3	0	Fl-RGG (Isologous)	IV	.25 (0.05)	–
		8	Fl-RGG (Isologous)	IV	1.25 (0.20)	0.75 (0.05)
			Fl/KLH	SC		
III	3	0	Fl-KLH	IV	0.45 (0.1)	0.78 (0.04)
		8	Fl-KLH	IV	1.5 (0.35)	0.76 (0.05)
			Fl-KLH	SC		

[a] Each of these values is the arithmetic mean of the maximum, reached after 2 weeks in all instances, anti-Fl concentrations found during the anti-Fl response of each of the rabbits in a particular group. The standard deviations are indicated in parentheses.

[b] The limit of detection of anti-Fl antibody in our assay was 0.1 mg/ml.

TABLE II. SUPPRESSION OF THE SECONDARY RESPONSE TO Fl BY Fl-RGG (AUTOLOGOUS)

Group	No. of rabbits	Time of infection (week)	Conjugate	Route of administration	Average maximum anti-Fl concentration in serum (mg/ml)[a]	Average maximum anti-KLH concentration in serum (mg/ml)
IV	5	0	Fl-KLH	SC	1.2 (0.15)	0.74 (0.03)
		8	Fl-RGG (autologous)	IV	<0.1[b,c]	0.77 (0.02)
			Fl-KLH	SC		
		16	Fl-KLH	SC	1.15 (0.30)	0.82 (0.04)
V	5	0	Fl-KLH	SC	1.15 (0.20)	0.73 (0.04)
		8	Fl-KLH	IV	1.30 (0.35)	0.71 (0.03)
			Fl-KLH	SC		

[a] Each of these values is the arithmetic mean of the maximum, reached after two weeks in all cases, anti-Fl concentrations found during the anti-Fl response of each of the rabbits in a particular group. The standard deviations are indicated in parentheses.

[b] The limit of detection of anti-Fl antibody in our assay was 0.1 mg/ml.

[c] A sixth rabbit gave a detectable response to the second injection yielding a maximum anti-Fl concentration of 0.3 mg/ml.

TABLE III. RABBIT ANTI-Fl RESPONSES TO Fl-CARRIER CONJUGATES

No. of rabbits	Conjugate	Moles of Fl per mole of carrier	Route of administration	Average maximum anti-Fl concentration in serum (mg/ml)[a]
8	Fl-KLH	85:1	SC	1.2 (0.2)
3	Fl-RGG (isologous)	8:1	SC	0.25 (0.05)
3	Fl-RGG (autologous)	8:1	SC	<0.1[b]
3	Fl-KLH	10:1	IV	0.45 (0.1)
3	Fl-RGG (isologous)	8:1	IV	0.25 (0.05)
3	Fl-RGG (autologous)	8:1	IV	<0.1

[a] Each of these values is the arithmetic mean of the maximum anti-Fl concentration reached in all instances 2 weeks after immunization found during the anti-Fl response of each rabbit in a particular group. The standard deviations are indicated in parentheses.

[b] The limit of detection of anti-Fl antibody in our assay is 0.1 mg/ml.

autologous IgG was not immunogenic even when given subcutaneously in complete Freund's adjuvant.

Discussion. These studies show that, in rabbits, either the primary or the secondary anti-Fl response to Fl-KLH may be suppressed by treatment with a nonimmunogenic hapten carrier conjugate Fl-RGG (autologous). The induced tolerance is due to the autologous carrier and is hapten-specific. Recent work has shown that the autologous nature of IgG is not sufficient for tolerogenicity. The mode of attachment of the hapten to the carrier is also critical for tolerance induction (11). The efficiency of autologous IgG as a tolerance inducing carrier, as compared to hemologous IgG may be due to an allotypic difference of these rabbit gamma globulins. Thus, an immune response against a foreign allotype may have rendered Fl-isologous rabbit gamma globulin immunogenic.

The induction of carrier-determined tolerance in unprimed animals appears to involve a cellular mechanism in which the receptors of the antigen binding cells (ABC) are blocked by persistently bound tolerogen (12). The recent observation (13) that, in rats, fluorescein presented as tolerogen remains on the surface of the lymphoid cell, is consistent with this interpretation.

We have also found that tolerance may be induced in primed animals, which, presumably, have a greater number of antigen-binding cells (ABC) specific for fluorescein than in umprimed animals. This observation suggests that the ABC of the primed animals, which are known to process a greater number of specific receptors on their surface (14), have been paralyzed. The observation that carrier determined tolerance is transient and requires the constant presence of the tolerogen may imply that receptor blockade is reversible in vivo as was recently

shown to be the case *in vitro* (15) when tolerance was broken. This mechanism may involve both T and B cells as has been demonstrated in a different system (16).

Finally, it should be emphasized that the ability to induce tolerance to a fluorescent hapten offers several distinct advantages: Firstly, it will permit direct visualization of the fate of the tolerogen *in vivo*. Thus, one can determine by a more simple technique than autoradiography, whether the tolerogen, in contrast to the immunogen, stays on the surface of the antigen binding cell. Secondly, receptor blockade of the anitigen binding cell by a fluorescent hapten will allow the separation of the tolerant cell (i.e., antigen binding cell with its receptor occupied by the tolerogen), by means of a cell sorter apparatus (17). If a relatively pure population of tolerant cells can be obtained then a number of studies on the function as well as the metabolism of these cells can be undertaken, thereby opening a rational approach to the cellular basis of tolerance.

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