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CROSS-SUPPRESSION OF SPECIFIC IMMUNE RESPONSES AFTER ORAL TOLERANCE

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Adult normal inbred mice rendered tolerant to OVA by previous oral exposure do not respond to intraperitoneal immunization with DNP-OVA in adjuvant. These tolerant mice also form less DNP-specific antibodies to DNP-KLH when immunized with mixtures of DNP-KLH and DNP-OVA, or less HGG-specific antibodies when immunized with cross-linked conjugates of OVA and HGG. These same procedures increased DNP-specific or HGG-specific responses in non-tolerant control mice.

The cross-suppression was ineffective, however, to inhibit already ongoing antibody responses.

It has been known for quite a few years that the ingestion of protein antigens may lead to the development of immunological tolerance (Dakin, 1829); Besredka (1909); Wells (1911); Wells & Osborne (1911); Gilmore (1912). The interest in this phenomenon has been recently revived (Andre et al, 1975); Thomas & Parrot (1974); David (1975); Vaz et al (1977); Hanson et al (1977, 1979a, b); Ngan & Kind (1978); Richman et al (1978); Miller & Hanson (1979). There is clear evidence that the tolerance resulting from the oral administration of antigen to adult animals results from the development of suppressor T cells (Richman et al, 1978); Ngan & Kind (1978); Miller & Hanson (1979).

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Although very effective to block the initiation of immune responses, feeding with the antigen has been observed to increase, rather than decrease antibody formation in previously immunized mice (Hanson et al, 1979a). This places a considerable limitation on the usefulness of oral tolerance for possible clinical applications.

The present experiments were undertaken to evaluate whether the specific immune suppression induced orally to one protein antigen might be used to suppress a second, unrelated immune response, through the introduction of specific ligand molecules or molecular conjugates. The tolerance induced orally is a carrier-specific T-cell effect (Vaz et al, 1977); Richman et al (1978). For example: mice given dinitrophenylated ovalbumin (DNP-OVA) orally are perfectly able to form anti-DNP antibodies when injected with DNP-hemocyanin. We wanted to test whether mice made tolerant to OVA administered orally – which develop OVA-specific suppressor T-cells (Richman et al, 1978) – would focus these suppressor cells on cells responding to an unrelated antigen – for example, DNP-hemocyanin – when exposed concomitantly to DNP-OVA. Or, whether mice rendered tolerant to OVA would form normal amounts of antibodies to a second protein when exposed to conjugates (cross-linked) of OVA with this protein.

MATERIAL AND METHODS

Mice. Adult female BDF1 mice (C57BL/6J x DBA/2J) were purchased from the Jackson Laboratory, Bar Harbor, Maine.

Antigens. Ovalbumin (OVA, grade V, Sigma, St. Louis, Mo), Keyhole limpet haemocyanin (KLH, Pacific Biomarine Supplies, Venice, Ca) and Human Gamma Globulins (HGG, Cohn Fr II, Sigma) were used as immunogens either as native proteins, after dinitrophenylation (Ovary & Benacerraf, 1963) or after cross-linking by glutaraldehyde (Avrameas, 1969). A conjugate of OVA and HGG, containing 4 μg OVA per 1 μg HGG was used throughout the study. The DNP conjugates of OVA and HGG contained respectively 9 and 84 DNP groups per mole (KLH assumed to weight 10^4 daltons).

Adjuvant. Al $(OH)_3$ gel was used as an adjuvant for immunization (Levine & Vaz, 1970). It was added to protein solutions immediately before the injection of the mixtures by intraperitoneal route into mice.

Tolerance induction by oral route. Tolerance was induced orally one week before primary immunization, by intragastric administration of 20 mg of antigen (OVA or HGG) in 0.5 ml of isotonic NaCl; mice were lightly anesthetized with ether and intubated with a premature infant feeding catheter (Nº 3131, Bard-Park, Rutherford, NJ).

Antibody assays. Antibody titers (ABC-33) were determined by modified Farr techniques, in which precipitation was achieved with polyethylene glycol (Creighton et al, 1973) instead of ammonium sulphate, used in concentrations which precipitated antigen-antibody complexes but left unbound antigen in the supernatant. HGG-specific and DNP-specific tests used respectively ^{125}I-HGG and ^{125}I-DNP-Mouse serum albumin (Sigma) as antigens. Details of the test concerning iodination, dilutions and determination of the ABC-33 were previously reported (Vaz et al, 1971); Hanson et al (1977).

RESULTS

As shown in Table 1 small doses of either DNP-OVA or DNP-KLH in Al $(OH)_3$ adjuvant were virtually equipotent for the induction of secondary DNP-specific antibody responses in BDF1 mice. When both immunogens were injected together, there was no evidence of antigenic competition, and the response was twice as intense.

TABLE I

The formation of DNP-specific antibodies in normal mice following immunization with DNP-ovalbumin, DNP-hemocyanin or both

Primary and secondary immunization[a] *(days 0 and 28)*	*Primary titers (day 14)*	*Secondary titers (day 35)*
DNP-OVA	4 ± 1[b]	55 ± 12
DNP-KLH	6 ± 2	41 ± 10
DNP-KLH + DNP-OVA	4 ± 1	113 ± 15

a = Primary and secondary immunization with 1 μg DNP-OVA or DNP-KLH, or 1 μg of both antigens, intraperitoneally, with 1 mg Al $(OH)_3$ adjuvant.

b = ABC-33 (mean ± standard error) in groups of 5 BDF1 mice.

As shown in Table II when the same antigens were injected into mice which had received previously 20 mg of OVA by oral route, the results were significantly different. No antibody responses were elicited by DNP-OVA, indicating total tolerance to this immunogen. The DNP-specific responses to DNP-KLH were not decreased; if anything, they were higher than those observed in normal mice. However, when to the immunizing dose of DNP-KLH was added DNP-OVA, the responses were significantly lower than those to DNP-KLH alone, or the resposes of normal mice to the mixture of the two antigens.

TABLE II

The formation of DNP-specific antibodies in mice previously tolerized to ovalbumin by oral route, following immunization with DNP-ovalbumin, DNP-hemocyanin, or both antigens

Primary and secondary immunization[a] *(days 0 and 28)*	*Primary titers (day 14)*	*Secondary titers (day 35)*
DNP-OVA	0 ± 0[b]	0 ± 0
DNP-KLH	6 ± 1	98 ± 26
DNP-KLH + DNP-OVA	1 ± 1	11 ± 1

a and b = as in Table I.

Mice tolerized with OVA 7 days before primary immunization (20 mg orally).

We attempted next to duplicate these results with a conjugate of two unrelated proteins – HGG and OVA – aggregated (cross-linked) with glutaraldehyde. To our surprise, HGG demonstrated to be very poorly immunogenic to BDF1 when injected in low doses with Al $(OH)_3$ adjuvant. Aggregation of HGG with glutaraldehyde by itself did not improve the immunogenicity. However, when HGG was cross-linked in the presence of OVA, forming a HGG:OVA-aggregate, substantial amounts of HGG-specific antibodies were formed. These results are shown in Table III.

TABLE III

The immunogenicity of human gamma globulin is increased by conjugation with ovalbumin

Primary and secondary immunization[a] *(days 0 and 28)*	*HGG-specific secondary titers (day 35)*
HGG	1 ± 1[b]
aggregated-HGG	1 ± 0
aggregated-HGG:OVA	126 ± 26

a = Primary and secondary immunization with 1 μg HGG, either as a native protein, or aggregated alone, or aggregated with OVA (1 μg HGG + 4 μg OVA/mouse).

b = ABC-33 (mean ± standard error) in groups of 5 BDF1 mice.

Although this was somewhat different from what we had planned, we investigated the effect of oral pretreatment with either HGG or OVA on the HGG-specific response to the HGG-OVA-aggregate. The results are shown in Table IV. Oral treatment with HGG decreased significantly, and oral treatment with OVA totally abolished the responses. The result obtained with oral HGG was particularly interesting, since it indicated that HGG was not merely functioning as a hapten, and could by itself initiate an immunologically specific event, such as oral tolerance.

TABLE IV

Oral tolerance to human gamma globulin or to ovalbumin reduces responses to the aggregate of the two proteins

Intragastric pretreatment (day-7)	*Primary and secondary immunization (days 0 and 28)*[a]	*HGG-specific secondary titers (day 35)*
Saline	aggregated-HGG-OVA	126 ± 26[b]
HGG 20mg	aggregated-HGG-OVA	25 ± 9
OVA 20mg	aggregated-HGG-OVA	0 ± 0

a and b = as in Table III.

In the next experiment two groups of mice were immunized to DNP-KLH in $Al(OH)_3$. One week after a second dose of DNP-KLH (day 28), mice in one of the groups were tolerized with 20 mg OVA orally; mice in the control group received saline intragastrically. Test bleedings performed on days 28 and 35 revealed that the titers of anti-DNP antibodies in the two groups did not differ significantly at this stage of the experiment, although they were slightly lower in the tolerant group. From day 35 to 41 all animals were given daily injections of 10 μg of DNP-OVA intraperitoneally, to investigate whether these injections would reduce the anti-DNP titers in the tolerant animals. However, bleedings made on days 42, 49 and 56 showed no significant variations; actually, there was no statistically significant variation in the titers assayed in this group from day 28 to day 56. On the other hand, as expected from normal mice, non-tolerant to OVA,

very significant increases in the anti-DNP antibodies were noted. The conclusion, therefore, was that DNP-OVA was not able to reduce already established anti-DNP antibody titers, although as shown in Table II, when tolerant mice were exposed to DNP-OVA and DNP-KLH at the same time, very significant reductions in the final titer of anti-DNP antibodies were noted. This might signify that suppressor cells induced by oral exposure to antigen, although efficient to block the increase in antibody titers which would result from a reexposure to antigen, are not efficient to interrupt ongoing antibody formation.

TABLE V

Failure to interrupt the formation of anti-DNP antibodies to DNP-KLH by repeated injections of DNP-OVA in mice tolerant to OVA

Primary-secondary immunization (days 0 and 21)[a]	*Oral tolerance to OVA (day 28)*[b]	*Intraperitoneal treatments (days 35-41)*	*DNP-specific antibody titers*[c]				
			(day 28)	*(day 35)*	*(day 42)*	*(day 49)*	*(day 56)*
DNP-KLH	no	DNP-OVA 10 μg/day	191 ± 50	242 ± 61	383 ± 118	801 ± 358	538 ± 132
DNP-KLH	yes	DNP-OVA 10 μg/day	106 ± 35	187 ± 72	104 ± 32	174 ± 33	141 ± 31

a = 1 μg DNP-KLH + 1 mg Al $(OH)_3$, intraperitoneally.

b = 20 mg of OVA intragastrically, or saline.

c = ABC-33 (mean ± standard error) in groups of 6 BDF1 mice.

DISCUSSION

Although they may be studied as isolated clonal events, specific immune responses result from a complex network of cell interactions. The initiation of immune responses, as well as their termination, are changes in the patterns of connectivity among lymphoid cells and their products. Antigen molecules represent one type of link which may physically connect previously unrelated cell types, and thus bring about mutual stimulation or inhibition; antibodies directed to idiotypic determinants are a second type of link (Eichman, 1974); Jerne (1974); Vaz & Varela (1978).

When hapten-protein conjugates bind to hapten-specific antibodies on the membrane of B cells, these cells become potential targets for the helper or suppressor influences of carrier-specific T cells. T and B cells which have been stimulated by different antigen molecules, which were either given separately to the same animal (Rajewsky et al, 1969), or, given to separate organisms and later transfered to a third compatible partner (Mitchinson, 1971), may be linked and cross-stimulate each other when connected by an antigen sharing determinants of the first two molecules. This third molecule, therefore, constitutes an appropriate ligant between previously unconnected cell populations, which once brought together, interact.

Similarly, it should be possible to bring together a population of suppressor cells and a population of responding cells that should be suppressed, by the introduction of an appropriate ligant molecule.

In the first set of experiments (Tables I and II) we have shown that the addition of DNP-OVA to an immunizing dose of DNP-KLH decreases the formation of anti-DNP antibodies in tolerant mice (ABC-33 of 11 ± 1 against 113 ± 15 in non-tolerant mice). In experiments not shown in the tables, we observed that a smaller degree of suppression could also be obtained when native OVA, instead of DNP-OVA was mixed with DNP-KLH, suggesting that some degree of unspecific suppression may also be involved in the process.

In the next set of experiments, we attempted to demonstrate similar events with two proteins. One of the proteins, however, HGG, showed to be non-immunogenic by itself; HGG-specific antibodies, however, could be easily produced when HGG was conjugated to OVA. The potentiation of immune responses by concomitant presentation of

weak and strong antigens is widely known in various situations: the production of antibodies to blood group antigens in birds (McBride & Shierman, 1971); the effect of mycobacteria in the response of guinea pigs to synthetic antigens (Katz & Benacerraf, 1972); effects of tuberculin on responses to diphteria toxoid (Humphrey & Turk, 1963); of tetanus toxoid, or burro red cells, on responses to sheep red cells (Rubin & Coons, 1971); of azobenzenearsonate-N-acetyl-L-tyrosine (ABAT) on delayed hypersensitivity to ABAT-red cells (Brunda & Raffel; 1979); to PPD increasing the antigenicity to tumour cells (Lachman & Sicora, 1978).

Oral tolerance to OVA or to HGG was able to inhibit subsequent HGG-specific antibody formation induced by HGG-OVA-aggregates. HGG however was a weaker tolerogen than OVA. The behavior of HGG in this system is reminiscent of the behavior of an auto-antigen: it is poorly immunogenic by itself, even when aggregated and injected in adjuvant, but it may elicit specific responses when conjugated to a strong immunogen.

The formation of IgE antibodies to OVA and ragweed antigen E may be blocked in mice treated with conjugates of these proteins with either autologous immunoglobulin, (Lee & Sehon, 1978), tolerogenic compounds such as D-GL (Liu et al, 1979), or other compounds such as polyethylene glycol (Lee & Sehon, 1978b) or pululan (Usui & Matuhasi, 1979). The suppression of IgE antibody responses is achieved more readily than the suppression of IgG responses (Lee & Sehon, 1978a), something also noted after oral tolerance (Vez et al, 1977) and in the partial tolerance induced by intravenous injections of OVA (Maia, Vaz & Vaz, 1974); Ishizaka (1976).

Finally, there is evidence that very large doses of a tolerated antigen may inhibit unrelated antibody responses. This has been shown in mice made tolerant to human albumin or bovine globulins in the neonatal period, and then immunized with OVA in the presence of large doses of these proteins (Liacopoulos & Herlem, 1968), a result which may derive from the production of unspecific suppressor factors (Gershon, 1975).

The central message, therefore, is that clonal connectivity is one of the basic properties of the lymphoid system, and that appropriate ligant molecules might be used to intervene in this connectivity. The present results suggest that oral tolerance may be used to generate suppressor cells to protein antigens in adult organisms, and then used to suppress the initiation or elevation of unrelated immune responses by treatment with ligant molecules linking these cells to the processes to be suppressed. We have been unable to show that these same treatments may decrease ongoing antibody formation.

RESUMO

.Camundongos adultos normais tornados imunologicamente tolerantes a ovoalbumina (OVA) por exposição oral não formam anticorpos antidinitrofenil (anti-DNP) quando imunizados com DNP-OVA, mas respondem normalmente à DNP-hemocianina (DNO-KLH). Entretanto, a adição de DNP-OVA à injeção de DNP-KLH reduz a formação de anticorpos anti-DNP em animais tolerantes à OVA, mas não em animais normais. Similarmente animais tolerantes à OVA formam menos anticorpos antiglobulina humana (HGG) quando imunizados com agregados (por glutaraldeído) de OVA e HGG. A tolerância oral é, portanto, capaz de inibir a indução de respostas imunes por um esquema de supressão-cruzada. Esse esquema, no entanto, não foi capaz de inibir respostas imunes já iniciadas.

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