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Cellular basis of persistent tolerance induced by an aggregate free heterologous immunoglobulin

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Summary. Attempts were made to break the tolerance of lymph node B cells to deaggregated human γ globulin. Using allotype-congenic mice, lymph node cells from virgin or tolerant (5 mg) CBA/Ig^b donors were transferred to normal CBA/Ig^a recipients and the proportion of responding donor B cells estimated 1 and 13 days later. The response of the non-tolerant virgin cells diminished with time but was still detectable at 13 days whereas the response of the tolerant cells was tenfold lower than normal cells at day 1 and was not detectable at 13 days. This functional deletion of tolerant cells was not reversed by enzymatic stripping of the immunoglobulin receptors before transfer, nor by removing T cells—which might have had a suppressor action.

In other experiments Ig^b mice were thymectomized or left as controls at various times before tolerance induction. Lymph node cells from these mice were transferred, together with non-tolerant Ig^a cells, to irradiated recipients. The cells from tolerant *thymectomized* donors strongly suppressed the response of non-tolerant cells, whereas the tolerant control cells showed no suppressor activity. It is considered that B-cell tolerance can be maintained by something other than receptor blockade, or active suppression—although the latter can arise in some circumstances.

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INTRODUCTION

Since the original finding by Dresser (1962) that the injection of aggregate-free proteins induce tolerance, considerable efforts have been made to discover the cellular lesion resulting in the lack of an antibody response. Most aggregate-free protein antigens induce tolerance primarily in T cells (Taylor, 1969) even at high antigen concentration (Rajewsky & Brenig, 1973). In contrast the induction of tolerance to protein antigens in mature virgin B cells seems to be restricted to antigens such as immunoglobulins (Chiller & Weigle, 1973; Scott, 1973; Taylor & Elson, 1974). Because the numbers of the corresponding antigen binding cells drop after injection of tolerogenic doses of deaggregated human γ globulin (HγG) it was considered (Louis, Chiller & Weigle, 1973) that the tolerance induced may reflect the clonal elimination of B cells as perceived by Burnet (1959). This view is currently debated. For example, Coutinho & Moller (1973; 1975) have proposed that mature virgin B cells can give only one response to antigen *viz.* a positive one and as a corollary to this hypothesis they question whether clonal elimination of B cells occurs. Instead, B cell unresponsiveness is envisaged as a reversible blockage of the antigen binding receptor by soluble antigen.

If clonal deletion does not take place then it would be expected that tolerant B cells might

eventually recover responsiveness after transfer to an appropriate recipient. Before testing this it is necessary to know how long virgin B cells are able to persist in the absence of antigen. Recently, we found the functional half life of virgin TNP-reactive lymphocytes to be about 1 week (Elson, Jablonska & Taylor, 1976). The value was obtained by transferring lymph node cells of one allotype to normal congenic mice bearing another allotype and measuring the proportion of donor B cells at intervals. This was done by making a further transfer to irradiated recipients and challenging with a TNP conjugate. The donor contribution to the antibody response declined with time in an approximately exponential fashion to give the functional half life. The present report describes the extension of this system to the study of B cell tolerance to HγG. No evidence could be found in non-thymectomized mice that tolerance was maintained either by blocking of surface receptors or by suppressor T cells. In adult-thymectomized mice on the other hand, a strong suppressor activity was found.

MATERIALS AND METHODS

Animals

Male and female CBA/H/Ig^b mice (referred to as Ig^b mice) were used as donors of virgin or tolerant lymph node cells depending on their previous immunological history. The congenic CBA/H mice (which bear the Ig^a allotype) were used as intermediate hosts and as irradiated recipients.

Antigens

In most cases tolerance was induced by injecting each mouse with 5 mg deaggregated human gamma globulin (HγG) Cohn Fractions II (Koch Light). Immediately before injection HγG was absorbed with mouse spleen cells and deaggregated by centrifugation for 2½ h at 100,000 *g* in a swing-out rotor. Only the material from the top third of each centrifuge tube was used. The mice were left between 10 and 12 days before use as cell donors, unless otherwise stated. The HγG used for immunization and in the antibody assay was further purified by elution from DE-23 (Whatman) in 0·02 M Tris HCl pH 9·0. Mice were immunized by intraperitoneal injection of 100 μg alum-precipitated HγG with pertussis as adjuvant.

Antibody assay

A radioimmunoassay was used, based on that described by Klinman & Taylor (1969), as described elsewhere (Elson & Taylor, 1974). Essentially HγG was coupled to bromacetyl cellulose. Aliquots of this were added to dilutions of the test sera, washed, and then incubated with ^{125}I-labelled purified anti-mouse immunoglobulin antibody, washed again and the radioactivity counted. Polyspecific sheep anti-mouse Ig antibody was used for estimates of the total antibody and anti-Ig^b allotype antibody for estimates of antibody bearing Ig^b allotype determinants. By comparison with results obtained with a standard purified anti-DNP (and DNP bromacetyl-cellulose), it was possible to estimate antibody concentration in test sera in terms of micrograms per millilitre.

Treatment with chymotrypsin

Washed cells (5×10^7/ml) were incubated in a balanced salt solution containing 0·05% chymotrypsin (Boehringer & Soehne) and 0·002% DNA ase (Sigma) for 30 min at 37°. They were washed twice and resuspended in balanced salt solution. The concentration of chymotrypsin used is twice that previously reported to remove the surface immunoglobulin from mouse cells (Elson, Taylor & Singh, 1973). The treated cells were found not to fluoresce after treatment with fluorescein labelled anti-mouse immunoglobulin although they were 95% viable as judged by trypan blue exclusion.

T cell depletion

Rabbit anti-mouse brain serum (Golub, 1971) was prepared, absorbed and its specificity for T cells tested as described previously (Elson & Taylor, 1975). Cells (5×10^7/ml) were incubated with a 1/50 dilution of anti-brain serum and a 1/20 dilution of agarose absorbed guinea-pig complement. After incubation the cells were washed twice.

Cell transfers and general plan of experiments

Virgin or tolerant Ig^b mice were killed by cervical dislocation. Cell suspensions were made from the superficial lymph nodes. Some of the virgin cells and some of the tolerant cells were treated with chymotrypsin. Virgin cells, chymotrypsinized virgin cells, tolerant cells or chymotrypsinized tolerant cells (8×10^7) were injected intravenously into CBA mice. Either 1 or 13 days later groups of these intermediate hosts were killed. As injected lymph node

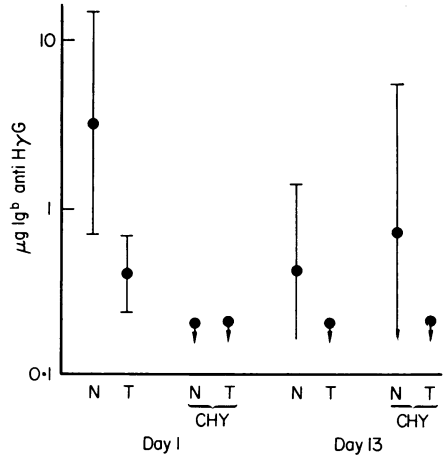

Figure 1. Functional deletion of tolerant B cells. Virgin, virgin chymotrypsinized, tolerant or tolerant chymotrypsinized Ig^b lymph node cells (8×10^7) were injected into CBA mice. One or 13 days later the CBA mice were killed and the response of the Ig^b cells then present was measured. The mean and standard deviation are shown.

cells distribute both to the lymph node and spleen (Zatz & Lance, 1970). These organs were pooled for each individual intermediate host. $2{\cdot}5 \times 10^7$ cells from such a pool were injected intraperitoneally into an irradiated (500 rad) CBA recipient, and this was repeated for each pool. The recipients were challenged 1 day after this cell transfer and bled 21 days later.

RESULTS

Persistent tolerance

The results of the first experiment are expressed in Fig. 1. It is evident that the capacity of the virgin cells to respond was diminished with time spent in the intermediate hosts although they still produced a detectable response after a sojourn of 13 days. In contrast the response of the tolerant cells was about tenfold less than the normal cells at 1 day and they gave no detectable response at 13 days. Neither normal nor tolerant chymotrypsinized cells responded at 1 day, presumably because the enzyme treatment interfered with the localization of the cells in the spleen and lymph node (Woodruff & Gesner, 1968) and so no cells of donor (Ig^b) origin would be transferred with the intermediate host's lymphoid cells to the irradiated recipient. By 13 days the normal chymotrypsinized cells responded as well as untreated cells but the tolerant chymotrypsinized cells gave no detectable response.

Table 1. Effect of tolerant cells on response of normal cells

Ig^b donor	Chymotrypsinization	Day	Anti-HγG ($\log_{10}$ μg/ml)
Normal	–	1	2·11 ± 0·18
Tolerant	–	1	1·95 ± 0·22
Normal	+	1	2·21 ± 0·05
Tolerant	+	1	1·93 ± 0·27
Normal	–	13	1·94 ± 0·34
Tolerant	–	13	2·04 ± 0·53
Normal	+	13	1·81 ± 0·04
Tolerant	+	13	2·04 ± 0·13

The tolerant cells may have failed to recover because of active suppressor influences. As a mixture of tolerant donor (Ig^b) cells and normal CBA cells from the intermediate host was transferred to the irradiated recipient it was possible to test if the tolerant cells suppressed normal cells, by measuring the total anti-HγG response of the irradiated recipient. The results are summarized in Table 1 and it is clear that the response of normal cells was not significantly affected by the presence of tolerant cells.

It is still possible that tolerance was maintained by suppressor cells which acted solely on the Ig^b population. In a further experiment T cell-depleted normal Ig^b lymph node cells, tolerant Ig^b cells or T cell-depleted tolerant Ig^b cells (10^7) were transferred to groups of irradiated CBA recipients together with CBA spleen cells (10^7) as a source of co-operating T cells. The mice were challenged with HγG and their Ig^b anti-HγG response measured 21 days later. The response of the recipients of tolerant cells was $-0{\cdot}8 \pm 0{\cdot}47$ ($\log_{10}$ μg Ig^b Ab/ml) of tolerant T cell depleted cells was $-0{\cdot}74 \pm 0{\cdot}46$ and of T cell-depleted normal cells was $0{\cdot}59 \pm 0{\cdot}78$. Thus tolerance was independent of suppressor T cells.

Infectious tolerance in adult thymectomized mice

Although we have not so far detected active suppressor effects of cells from mice shortly (10–12 days) after injection of tolerogen, it is reported that suppressor activity is found if mice are left for some time after injection of tolerogen (Benjamin, 1975). Accordingly, an experiment was set up to test for suppressor activity of lymph node cells from mice

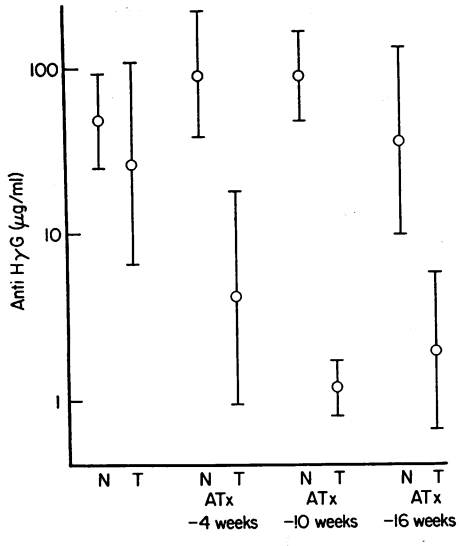

Figure 2. Effect of lymph node cells from tolerant Ig^b mice or tolerant adult thymectomized Ig^b mice on response of normal cells. Mice were adult thymectomized at the times shown before injection of tolerogen. Thirty-seven days after injection of deaggregated HγG their lymph node cells and those of controls were assayed for their capacity to suppress the anti-HγG response of normal CBA spleen cells.

exposed to tolerogen 37 days previously. In addition, because there is evidence that the generation of suppressor T cells is impaired in adult thymectomized mice (Kerbel & Eidinger, 1972; Nachtigal, Zan-Bar & Feldman, 1975; Basten, Miller & Johnson, 1975), the effect of tolerogen in adult thymectomized mice was tested. A pool of 6–8-week-old Ig^b mice was divided into groups which were thymectomized at 16, 10 or 4 weeks before injection of tolerogen or left to serve as controls. Half the mice in each group were injected with 4 mg deaggregated HγG per mouse. Thirty-seven days later lymph node cells from each mouse were collected. Two $\times 10^7$ cells from a donor were transferred to an irradiated (500 rad) CBA recipient together with 10^7 CBA spleen cells and this was repeated for each donor. The recipients were challenged with HγG and their total anti-HγG antibody measured 21 days later. The results are shown in Fig. 2 and it can be seen that only the cells from the tolerant *adult thymectomized* mice suppressed the response of normal cells.

The suppressor activity of lymph node cells taken from mice 12 days after injection of tolerogen into normal Ig^b mice or Ig^b mice adult thymectomized 16 weeks previously was tested. As can be seen from Table 2 the response of normal cells, as judged by the total anti-HγG response, was not significantly affected by the presence of tolerant cells from either normal or adult thymectomized donors although the latter were tolerant as judged by their depressed Ig^b anti-HγG response as compared with cells from non-tolerant controls.

DISCUSSION

These observations extend our previous work on the functional half life of virgin B cells (Elson, Taylor & Jablonska, 1976) by showing that cells taken from mice tolerant to HγG remain unresponsive after a sojourn of two functional half-lives in otherwise untreated intermediate hosts and thus that functional deletion does occur. There seems little doubt that lym-

Table 2. Lack of suppressive effect of lymph node cells from mice 12 days after injection of deaggregated HγG

Ig^b donor	Tolerogen injected day 0	Anti-HγG ($\log_{10}$ μg/ml)	Ig^b anti-HγG ($\log_{10}$ μg/ml)	Ig^b anti-HγG significance of difference
1 Normal	–	2·17 ± 0·37	1·28 ± 0·32	1–2
2 Normal	+	1·69 ± 0·70	0·14 ± 1·02	$t = 2{\cdot}6$ $P < 0{\cdot}05$
3 Adult thymectomized	–	1·93 ± 0·12	0·35 ± 1·18	3–4
4 Adult thymectomized	+	1·92 ± 0·29	−0·92 ± 0·19	$t = 2{\cdot}5$ $P < 0{\cdot}05$

Irradiated CBA recipients were injected with 10^7 lymph node cells from Ig^b donors as shown and 10^7 CBA spleen cells. They were challenged the following day and their total and Ig^b anti-HγG response measured a further 21 days later.

phocytes can be blocked by hapten-immunoglobulin conjugates (Aldo-Benson & Borel, 1974) so that they are unable to react to challenge with other conjugates of the same hapten on a different carrier. In this situation the unresponsiveness can be reversed either by incubating the putatively tolerant cells *in vitro* for 48 h (Aldo-Benson & Borel, 1976) or with a polyclonal B cell activator (Moller, Gronowicz, Persson, Coutinho, Moller, Hammarstrom & Smith, 1976). Although these authors did not formally exclude that these unresponsive states were maintained by active suppressor influences, serious consideration needs to be given to the idea that the functional deletion of B cells is merely the result of persistent blocking by tolerogen. Cells bearing hapten-immunoglobulin conjugates have been reported to persist for some weeks in tolerance induced with hapten-immunoglobulin conjugates by some authors (Aldo-Benson & Borel, 1974) but not by others (Scott, 1976). There is no obvious explanation for this discrepancy but we suggest that it may be related to the fact that some virgin B cells have a turnover rate of 48 h whereas others have a lower turnover rate similar to that of primed B cells (Strober, 1975; Strober & Dilley, 1973). It is difficult to see how tolerogen bearing cells of the former type could persist and if such virgin cells do have a certain intrinsic capacity to divide before they die, as seems to be true for TNP reactive B cells in mice (Elson, Taylor & Jablonska, 1976) then tolerance could not be maintained by blocking alone after transfer of tolerant cells to an antigen free environment.

Two conclusions force us to the conclusion that B cell tolerance can be maintained by something other than blocking. Firstly, in spite of the fact that chymotrypsin removed surface immunoglobulin from B cells and presumably removed HγG from the antigen binding receptors, responsiveness was not restored to tolerant cells. Secondly, since multivalent hapten HγG conjugates are rapidly lost from antigen binding cells *in vitro* (Wilson & Feldmann, 1972) a paucivalent antigen such as HγG would be expected to be retained even less well on the antigen binding receptors (Klaus, 1975). It should be emphasized that the present results were obtained with tolerant lymph node cells since we have had difficulty transferring tolerance with spleen cells (Taylor & Elson, 1974; Elson & Taylor, 1975), whereas the results of the other workers quoted were obtained with spleen cells.

There remains the possibility that tolerance was maintained by active suppressor mechanisms. This seems unlikely in view of the finding that tolerant cells did not affect the response of normal cells. However, it is not completely excluded because suppressor influences could act specifically on the tolerant Ig^b population through allotypic or idiotypic determinants. Against this, it was found that tolerance was not reversed by T cell depletion of tolerant cells. Moreover, the system for detecting 'infectious tolerance' was shown to be valid because cells from tolerant adult-thymectomized Ig^b mice suppressed the response of normal CBA cells.

The capacity of HγG tolerant cells to suppress the response of normal cells seems to be variable. For example, no suppressive effects were detected by some authors (Chiller, Louis, Skidmore & Weigle, 1974; Zolla & Naor, 1974), a finding in agreement with those described here. In contrast, other authors find either a transient suppression (about 1–3 weeks after administration of tolerogen (Basten, Miller & Johnson, 1975; Doyle, Parks & Weigle, 1976) or suppression appearing only 35 days and later after injection of tolerogen (Benjamin, 1975). Here, suppression was found 37 days after injection of tolerogen, but only in mice adult thymectomized some time previously. This does not necessarily conflict with the finding of impaired generation of suppressor T cells shortly after adult thymectomy in human serum albumin or HγG tolerant mice (Nachtigal, Zan-Bar & Feldmann, 1975; Basten, Miller & Johnson, 1975). Instead it may point towards the existence of a separate population of suppressor cells. At least two populations of suppressor T cells have been distinguished by their Ly phenotypes (Feldmann, Beverley, Woody & McKenzie, 1976; Cantor & Boyse, 1976) and/or by their differential susceptibility to adult thymectomy (Burns, Marrack, Kappler & Janeway, 1975; Wood, Asherson, Mayhew, Thomas & Zembala, 1977). As the net effect of the total T cell population is dependent on the helper/suppressor ratio (Cantor, Shen & Boyse, 1975; Herzenberg, Okumura & Metzler, 1975) then the increased suppressor activity in adult thymectomized mice could be due to the loss of helper T cells which occurs some time after adult thymectomy (Janeway, 1975) and the persistence of a suppressor population. Whatever the nature of this suppressor cell it seems clear that tolerance can persist in the absence of its activity.

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