SUPPRESSOR T CELL MEMORY

II. The Role of Memory Suppressor T Cells in Tolerance to Human Gamma Globulin*

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The precise mechanism(s) of immunological tolerance to both self- and non-self-antigens remains unclear, despite substantial advances in the understanding of cellular and molecular immunology over the past three decades. Current hypotheses may be divided into two broad (but not necessarily mutually exclusive) categories: (a) those involving direct, antigen-induced clonal inactivation of specific T and/or B cells, and (b) those in which suppressor T cells (Ts)[1] are held to be responsible for specific unresponsiveness. The first category includes classical clonal deletion as described originally by Burnet (1) and modified subsequently by Bretscher and Cohn (2) in their "two signal" hypothesis. According to the latter, contact of an antigen-reactive cell with antigen alone (signal one) in the absence of a second triggering signal from a collaborating cell (signal two) leads to irreversible inactivation of the cell concerned. Related to this are certain variations (3, 4) of Lederberg's original hypothesis of self-tolerance (5) which stated that developing lymphocytes are more sensitive than mature cells to antigen-induced inactivation. In some in vitro models receptor blockade has also been invoked as a mechanism for clonal inactivation (6), although this appears to be reversible (7) and its significance in vivo is uncertain.

In the second category of hypotheses, antigen-specific (or in some cases idiotype-specific) suppressor cells are held to be responsible for initiating and maintaining tolerance. Although Ts have been shown to be present in a wide variety of unresponsive states (8–13), their precise role in the induction and/or maintenance of unresponsiveness remains controversial. One of the major reasons for this uncertainty is the repeated observation that the kinetics and magnitude of suppressive activity do not parallel the time course and degree of tolerance (14–16). Thus some investigators have come to regard the presence of Ts as a regulatory epiphenomenon of little or no relevance to the mechanism of immunological unresponsiveness (15, 17, 18).

In this communication, Ts specific for the protein antigen human gamma globulin (HGG) have been shown to exist in two distinct functional states in tolerant animals, effector cells and memory cells. The activity of the former is readily apparent in standard adoptive mixing experiments shortly after tolerance induction, but their

presence is transient by comparison with the duration of the tolerant state. Memory Ts, on the other hand, do not express effector functions in adoptive transfer unless they are first reactivated by secondary antigenic stimulation. Although these cells are functionally latent in the tolerant animal, they are long-lived and capable of inducing rapid reexpression of effector activity after immunogen challenge. It will be argued that the physiological characteristics of memory Ts make them suitable candidates for a central role in the etiology of tolerance not only to HGG, but possibly also to self-antigens.

Materials and Methods

Animals. Male and female CBA/Ca/T6 mice were used. The strain was originally obtained from the Medical Research Council Animal Breeding Centre, Carshalton, England, and then maintained at the University of Sydney.

Antigens. HGG, Horse erythrocytes (HRC), and dinitrophenyl HGG (DNP$_9$HGG) were prepared as described previously (19).[2] The hapten, fluorescein (FLU) was purchased in the form of the isothiocyanate (isomer I) from BDH, Poole, England, and coupled to HGG or keyhole limpet hemocyanin (KLH) by the method of Nairn (20) to give the conjugates FLU$_9$HGG and FLU$_{3.2}$KLH.

Immunization. Hapten and carrier priming were carried out by intraperitoneal injection of the relevant alum-precipitated carrier together with 2 × 10^9 heat-killed *Bordetella Pertussis* organisms (Commonwealth Serum Laboratories, Melbourne, Australia) per mouse. The amount of each given was HGG, 500 µg, and DNP.KLH and FLU.KLH, 250 µg. Animals were immunized to HRC by intraperitoneal injection of 4 × 10^8 washed erythrocytes. Spleen cells for adoptive transfer were obtained from immune animals 4 wk or more after priming.

Tolerance Induction. Tolerance was routinely induced in adult mice by a single intravenous injection of 3 mg HGG that had been deaggregated by ultracentrifugation as described previously (19). In one group of experiments, lower doses were used (see Results).

For induction of tolerance before birth, pregnant mothers were injected intraperitoneally with 3 mg of deaggregated HGG (dHGG) at day 7 of gestation. After delivery, the offspring were suckled by noninjected foster mothers to eliminate the possibility of transfer of HGG via the milk.

Tracer Studies with ^{125}I-HGG. HGG was labeled with ^{125}I according to the chloramine-T method, as modified by Byrt and Ada (21). For administration to the pregnant mice, ^{125}I-HGG was added to unlabeled HGG before deaggregation. A total of 3 mg of dHGG with a specific activity of ~250,000 counts/min per mg of dHGG (as measured in a gamma counter [LKB-Wallac 80000; LKB-Producter AB, Stockholm, Sweden]) was then injected intraperitoneally into each pregnant mouse on day 7 of gestation. The offspring were killed at birth and checked for radioactivity.

Anti-HGG Antibodies. Antibodies to HGG were measured by a sensitive hemagglutination assay using human anti-D antiserum coupled to Rh-positive erythrocytes as described previously (19).

Anti-Ia^k Serum. Anti-Ia^k (A.TH anti-A.TL) was kindly provided by Professor I. F. C. McKenzie, Transplantation and Cancer Research Centre, University of Melbourne, Victoria, Australia; its preparation and use have been described elsewhere (22).

Cell Transfers. Recipient mice were exposed to 700 rad whole-body iradiation from a ^{60}Co source. The preparation and transfer of spleen cells were carried out as described previously (19).

Plaque-forming Cell Assay. Antibody-forming cells were detected by the method of Cunningham and Szenberg (23). Hapten-coated sheep erythrocytes (SRBC) were prepared by

[2] Loblay, R. H., H. Pritchard-Briscoe, and A. Basten. Suppressor T cell memory. I. Induction and recall of HGG-specific memory suppressor T cells and their role in regulation of antibody production. Manuscript submitted for publication.

incubation of washed erythrocytes with DNP- or FLU-conjugated F(ab')$_2$ rabbit IgG anti-SRBC antibody. Anti-HRC PFC were detected by assay with untreated HRC.

Statistical Analysis. Probabilities were determined using a two-tailed Student's t test.

Results

Primary Helper and Suppressor Activity in Tolerant Mice. It was shown previously that a mixed population of lymphocytes from HGG-immune animals containing both carrier-specific T helper (Th) and Ts cells can be made to exhibit either helper or suppressor function in an adoptive hapten-carrier system, depending on the number of carrier-specific cells transferred (24).[2] This observation was used here to document the activity of Th and Ts effector cells at various intervals after tolerance induction with dHGG in virgin mice. Helper activity was measured by transferring 5×10^6 spleen cells from tolerant mice together with 5×10^6 spleen cells from animals primed to DNP.KLH; the recipients were challenged with DNP.HGG, and their anti-DNP PFC responses assayed 7 d later. Suppressor cell function was assayed in a similar system by transferring 2.5×10^7 tolerant spleen cells into irradiated hosts together with 5×10^6 DNP-primed spleen cells and 5×10^6 helper cells from mice primed to HGG 3 or more wk previously. Controls received the same numbers of hapten- and carrier-primed cells, but 2.5×10^7 normal spleen cells were substituted for the tolerant cells. All recipients were challenged with DNP.HGG at the time of transfer, and splenic anti-DNP PFC responses were compared 7 d later.

The results from one such experiment are shown in Fig. 1. A small background response was seen in the helper assay (presumably due to the presence of hapten-specific Th [25]), but at no stage after tolerance induction was significant help above this level detectable. In the current and subsequent experiments, this was taken as evidence of carrier-specific tolerance. Primary suppression was consistently maximal between days 7 and 21, and began to wane by the 4th wk after tolerance induction, and was no longer detectable by day 35–40 in most experiments.

Secondary Suppression Induced by Reexposure of Tolerant Mice to dHGG. In previous studies it was shown that HGG-immune animals possess long-lived memory Ts specific for HGG (24).[2] The latter cells can be selectively restimulated by exposing immune animals to a "tolerizing" dose of dHGG 3 or more wk after priming. When this is done, the ensuing secondary suppressive effect displays the hallmarks of a classical anamnestic response, with acceleration, heightening, and prolongation of suppression (24).[2] To determine whether the presence of memory Ts could be detected in a similar
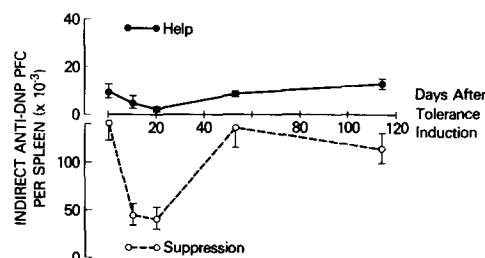

Fig. 1. Primary help and suppression after tolerance induction. Each point represents the geometric mean plaque-forming cells (PFC) ± SE obtained from groups of six irradiated recipients. The control response against which suppressor activity may be assessed was taken as the number of anti-DNP PFC observed in the presence of virgin spleen cells (O) at the origin (day 0).

manner in tolerant animals, mice tolerized 60 d previously were restimulated with a second injection of dHGG, and the capacity of their spleen cells to suppress a collaborative anti-hapten response to FLU.HGG was assayed in adoptive transfer 7 d later (Fig. 2, experiment I). This secondary suppression was compared with the primary suppressive activity generated in virgin control mice given dHGG at the same time. The results showed that secondary suppression by spleen cells (2.5×10^7) from dHGG-boosted tolerant mice (group 3 vs. 1) was considerably more potent than the suppression generated by a primary tolerizing injection of dHGG (group 2 vs. 1), thereby confirming the presence of memory suppressor cells in tolerant as well as immune animals.

To determine whether Ts memory in tolerant animals was long-lived, mice were boosted with 3 mg dHGG 135 d after tolerance induction, and their suppressive capacity 7 d later was compared in transfer with that of spleen cells from virgin mice given a primary injection of dHGG at the same time (Fig. 2, experiment II). The results again showed significant augmentation of the suppressive effect in previously tolerant animals after reexposure to dHGG (group 3 vs. 2) by comparison with primary suppression (group 2 vs. 1).

Reactivation of Memory Ts in Tolerant Animals by Immunogen Challenge. Maintenance of a tolerant state is usually assessed by demonstrating unresponsiveness to challenge with antigen in immunogenic form. If memory Ts do indeed play an important role in the maintenance of tolerance to HGG, then it would be predicted that immunogen challenge should stimulate a secondary Ts response in HGG tolerant animals after primary suppression had waned. To test this, mice rendered tolerant 70 d previously
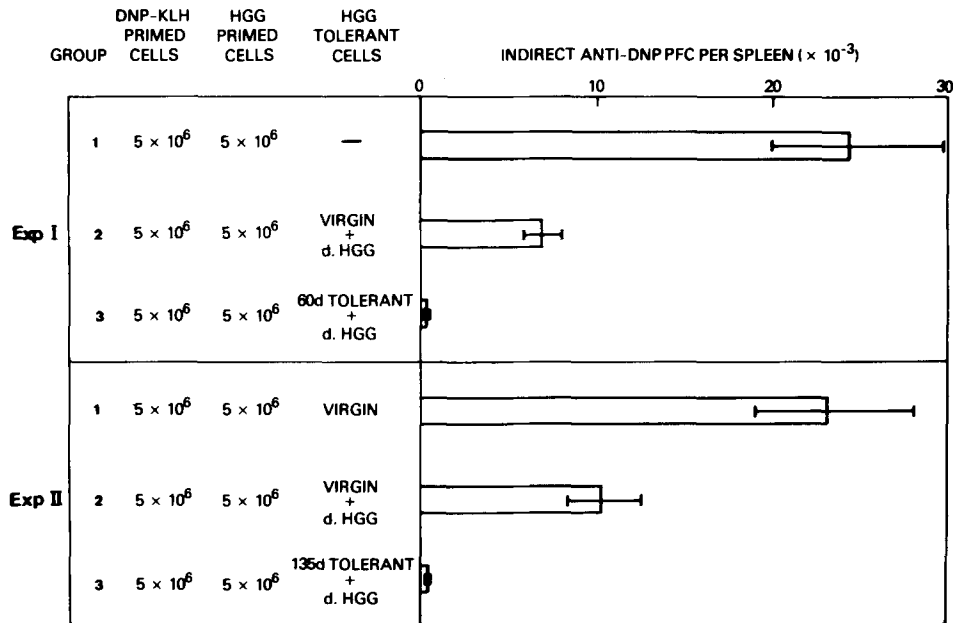

Fig. 2. Augmented secondary suppression in tolerant mice reexposed to dHGG. Each histogram represents the geometric mean PFC ± SE of six irradiated recipients per group. In addition to 5×10^6 hapten- and carrier-primed cells, recipients were given 2.5×10^7 spleen cells from: virgin donors given a primary tolerizing dose (3 mg i.p.) of dHGG (groups 2 and 5); tolerant donors boosted with dHGG at the same time (groups 3 and 6); virgin donors never exposed to dHGG (group 4).
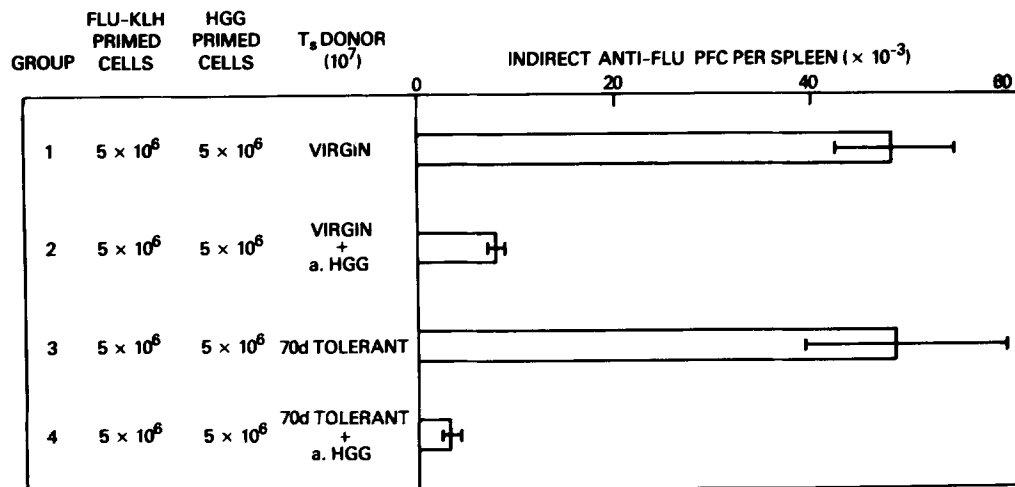
Fig. 3.   Augmented secondary suppression in tolerant mice challenged with aHGG. Each histogram
represents the geometric mean PFC ± SE of six recipients per group. Suppressed responses in groups
2 and 4 were significantly different ($P < 0.05$).

and virgin controls were challenged with alum-precipitated HGG (aHGG) and
assayed for suppressive activity 8 d later. At that time, primary suppression was no
longer detectable in the 70-d-tolerant donors (Fig. 3, group 3 vs. 1). As expected from
previous data (19, 24), primary immunization of virgin mice with aHGG resulted in
marked suppression (group 2 vs. 1), but by comparison, the degree of suppression
induced in tolerant animals challenged with immunogen at the same time was
significantly augmented (group 4 vs. 1).

Kinetics of Secondary Suppression in Tolerant Animals Challenged with Immunogen.   The
augmented secondary suppression seen after immunogen challenge of tolerant animals
was a highly reproducible observation, and suggested that an anamnestic Ts response
had indeed been stimulated. To confirm this, evidence was sought for an accelerated
onset and prolonged duration of the secondary suppressive effect following exposure
to aHGG.

The kinetics of primary and secondary effector Ts appearance were studied by
challenging virgin controls and 56-d-tolerant mice, respectively, with a standard dose
of aHGG at varying intervals before adoptive transfer. Suppression of the collaborative
anti-hapten response was assayed in the usual manner, and the results are depicted in
Fig. 4. Two important observations were made in this experiment. Firstly, the onset
of suppression in response to immunogen challenge was accelerated by 2 d in tolerant
mice compared with virgin mice immunized at the same time, which is consistent
with the presence of Ts memory in the tolerant donors. Secondly, the early peak of
helper activity seen at day 2 after primary immunization of virgin animals was absent
in the tolerant donors challenged with immunogen. This may represent either direct
suppression of Th activation, or the masking of Th activity in the assay system due to
suppression of the hapten-sensitive B cells.[2,3]

To determine whether secondary suppression in tolerant animals was of increased
duration, 56-d-tolerant and virgin mice were challenged with aHGG and left for a

[3] Loblay, R. H., H. Pritchard-Briscoe, and A. Basten. T cell-dependent suppression of antibody
production. II. Target cells and mechanism of action. Manuscript submitted for publication.
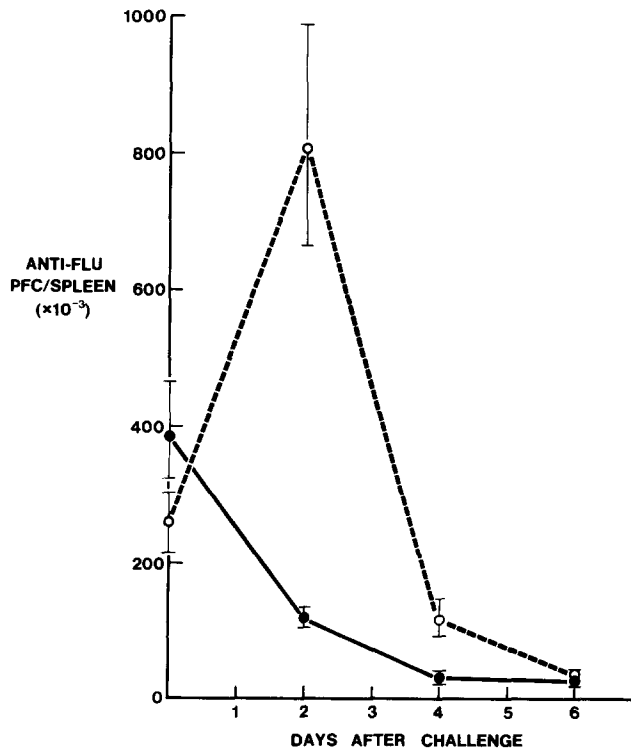
Fig. 4. Accelerated appearance of suppressive activity in tolerant mice after aHGG challenge. Each point represents the geometric mean PFC ± SE of six recipients per group. Recipients were given 5 × 10⁶ hapten- and carrier-primed cells in addition to 2.5 × 10⁷ cells from either virgin mice (○) or tolerant mice (●) challenged with aHGG at various intervals before transfer as indicated on the abscissa.
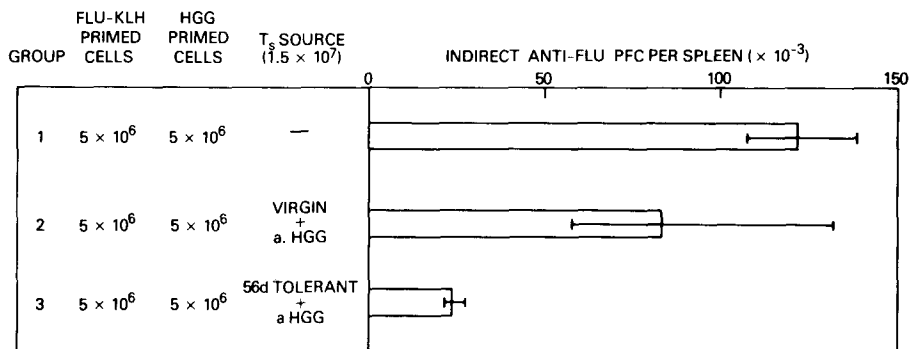
Fig. 5. Prolonged duration of suppression in tolerant mice after aHGG challenge. Each histogram represents the geometric mean PFC ± SE of six recipients per group.

further 50 d, at which time their spleen cells were removed and assayed for suppression in the standard adoptive transfer system. Fig. 5 shows that whereas primary suppression was barely detectable (group 2 vs. 1), highly significant suppression was still demonstrable with cells from the tolerant donors 50 d after immunogen challenge (group 3 vs. 1). Taken together, the above results strongly suggest that challenge of

tolerant animals with antigen in immunogenic form reactivates a population of memory Ts generated by the original tolerogenic stimulus.

Generation of Ts Memory With Low Doses of dHGG. To determine the dose of deaggregated antigen required for generation of Ts memory, groups of mice were given dHGG in doses varying from 1 μg to 3 mg as a single intraperitoneal injection. These animals, along with virgin controls, were challenged 42 d later with a standard dose of aHGG designed to reactivate putative Ts memory cells; their spleen cells were removed 5 d later and assayed for effector Ts activity in adoptive transfer, as before. As expected, modest primary suppression was observed with cells from virgin animals given aHGG 5 d previously (Fig. 6, group 2 vs. 1), and highly significant augmentation of the suppressive effect was seen in animals previously given 3 mg dHGG (group 5 vs. 1 and 2). Such augmentation, though less marked, was also observed in animals given a primary dHGG injection of 10 or 100 μg (groups 3 and 4 vs. 1 and 2). In other experiments, a small but significant degree of augmentation was seen with as little as 1 μg dHGG given before immunogen challenge (data not shown). Thus a single low dose of dHGG appears to be capable of generating Ts memory which may be readily recalled by subsequent immunogen challenge.

By contrast, primary suppression was not observed 10 d after a single injection of 100 μg dHGG, but is readily detectable after two or three injections of the same dose at three weekly intervals (Fig. 7). This emphasizes the point that Ts memory may be effectively generated in the absence of a detectable primary Ts response.

Generation of Primary Suppression in Mice Tolerized to HGG In Utero. The foregoing experiments indicate that both effector and memory Ts are readily induced in adult mice tolerized with HGG, but their importance in the development and maintenance of self-tolerance does not necessarily follow. To test whether tolerance induction during fetal development is accompanied by generation of effector and memory Ts, pregnant mice were injected intraperitoneally with dHGG on day 7 of gestation, and their offspring were tested for both tolerance and suppressive activity in adoptive transfer at various times after birth. It was shown by using ^{125}I-labeled HGG that the average amount of dHGG transferred to the neonatal mice before birth was ~1% of the maternal dose, i.e., 30 μg.

The antibody response of mice to a challenge dose of aHGG was tested at various

Group	FLU-KLH primed cells	HGG primed cells	OTHER (2.5×10^7)	INDIRECT ANTI-FLU PFC PER SPLEEN ($\times 10^{-3}$)
1	5×10^6	5×10^6	VIRGIN	
2	5×10^6	5×10^6	VIRGIN + aHGG	
3	5×10^6	5×10^6	10 μg dHGG + aHGG	
4	5×10^6	5×10^6	100 μg dHGG + aHGG	
5	5×10^6	5×10^6	3000 μg dHGG + aHGG	

FIG. 6. Augmented secondary suppression in animals previously exposed to a low dose of dHGG. Each histogram represents the geometric mean PFC ± SE of six recipients per group.
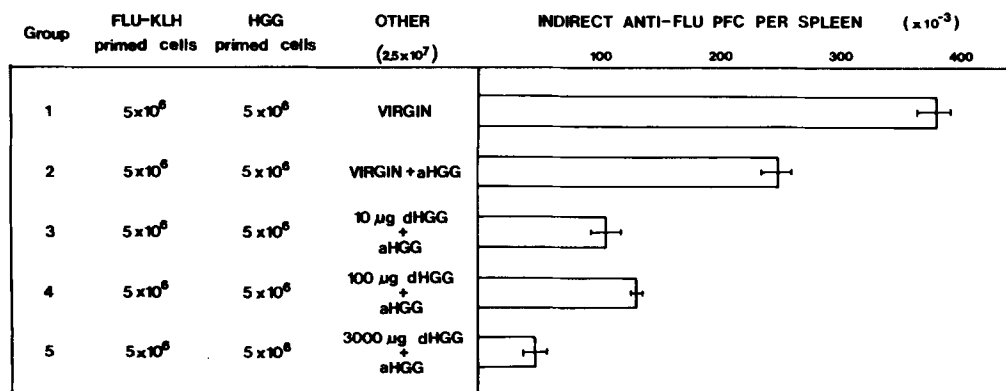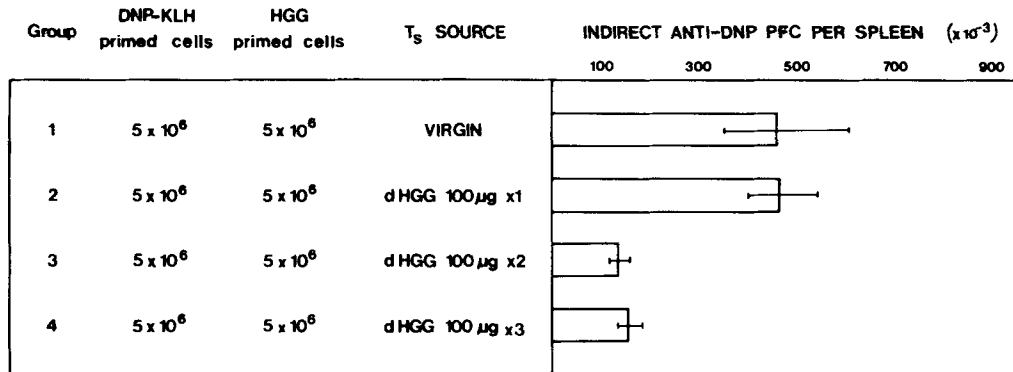
Fig. 7. Primary suppression generated by repeated low doses of dHGG. Each histogram represents the geometric mean PFC ± SE of six recipients per group.
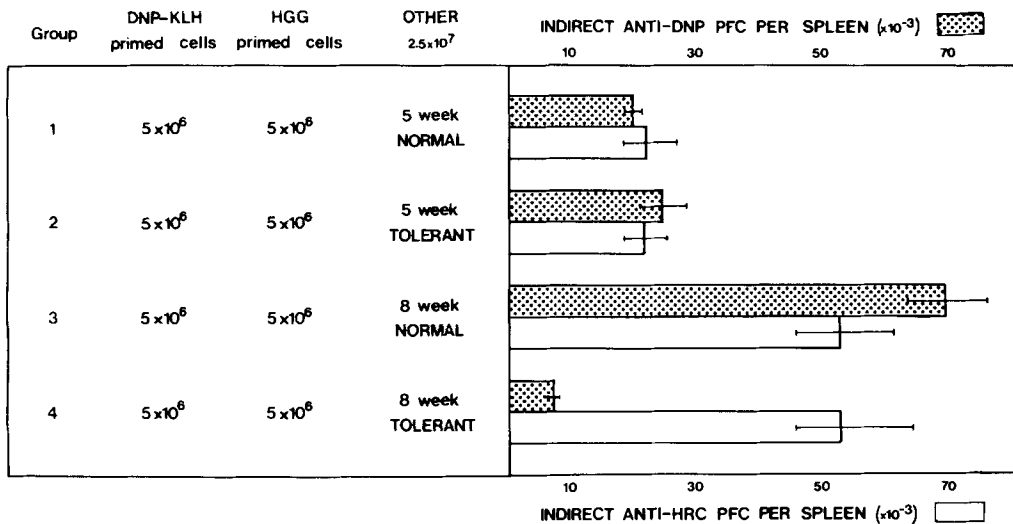

Fig. 8. Specific primary suppression in 8-wk-old mice exposed to dHGG in utero, emerging after the disappearance of nonspecific neonatal suppression. Each histogram represents the geometric mean PFC ± SE of six irradiated recipients.

times after birth by a haemagglutination assay. Mice exposed to dHGG in utero were completely tolerant at 4 wk of age, whereas age-matched controls produced anti-HGG titres ~10-fold less than the adult response. The tolerant state was gradually lost over the next month and full responsiveness appeared between the second and third months of life (data not shown).

To determine whether specific suppression had been generated in mice tolerized by prenatal contact with dHGG, they were used as cell donors in adoptive transfer experiments at various times after birth. One such experiment is shown in Fig. 8. At 5 wk of age, spleen cells from both normal and tolerant mice produced significant suppression of the adoptive antibody response compared with 8-wk-old controls (Fig. 8, groups 1 and 2 vs. 3). This was a non–antigen-specific effect, since the responses to HRC were as suppressed as those to DNP-HGG, and did not depend on prior exposure to antigen. Antigen-specific primary suppression was consistently demonstrable by 6 wk of age (at which time nonspecific suppression by neonatal cells had

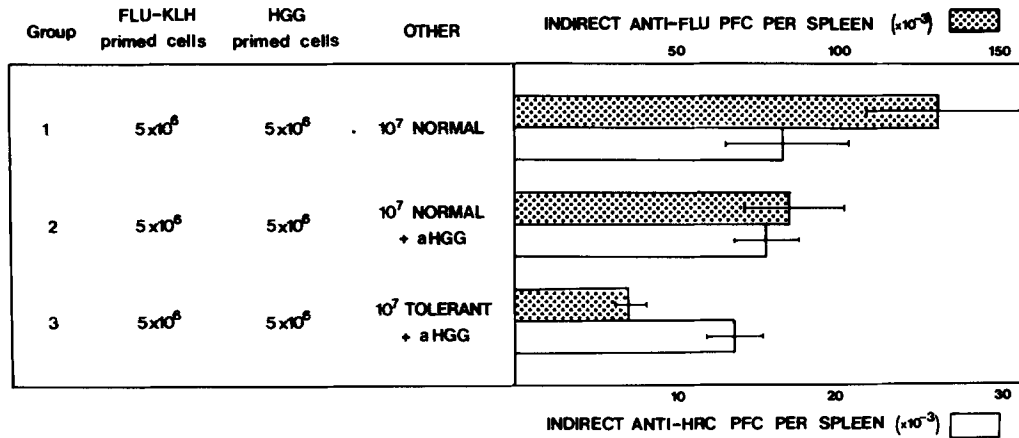| Group	FLU-KLH primed cells	HGG primed cells	OTHER	INDIRECT ANTI-FLU PFC PER SPLEEN (×10⁻³)
1	5×10⁶	5×10⁶	. 10⁷ NORMAL	
2	5×10⁶	5×10⁶	10⁷ NORMAL + aHGG	
3	5×10⁶	5×10⁶	10⁷ TOLERANT + aHGG	

INDIRECT ANTI-HRC PFC PER SPLEEN (×10⁻³)

Fig. 9. Augmented, antigen-specific secondary suppression after aHGG challenge of mice exposed to dHGG in utero. Each histogram represents the geometric mean PFC ± SE of six irradiated recipients.

waned) and persisted until ~12 wk of age, with a maximum at ~8 wk (Fig. 8, group 4 vs. 3).

Generation of Memory Suppression In Utero. If suppressor cells are responsible for the maintenance of self-tolerance throughout adult life, then it might be predicted that exposure to antigen in utero should generate long-lived memory Ts in addition to effector cells responsible for primary suppression. The experiment shown in Fig. 9 was designed to test this hypothesis. 6-wk-old mice that had been exposed to dHGG on day 7 of gestation were boosted with aHGG along with age- and sex-matched control animals never previously exposed to HGG, and their spleen cells were assayed for suppressive capacity in adoptive transfer 6 d later. Suboptimal cell numbers (10⁷) were used to render the assay system more sensitive to differing degrees of suppression. Cells from control mice given a primary challenge with aHGG exhibited modest specific suppression of the standard anti-hapten antibody response (Fig. 9, group 2 vs. 1). By comparison, suppression by cells from aHGG-challenged mice previously exposed to dHGG in utero was significantly enhanced (group 3 vs. 2); this effect was antigen-specific since no suppression of the anti-HRC response was observed. The augmented secondary suppressive effect was taken as evidence of the presence of long-lived Ts memory cells in animals exposed to dHGG during fetal life. By testing at various times, memory suppression could be demonstrated from 6 wk after birth up to the age of 6 mo, but by 10 mo this effect was no longer observed.

In all the experiments described above, similar results were obtained regardless of whether the neonatal mice were suckled by their natural mothers or fostered by nontolerized mothers. Thus, exposure to a tolerogen such as dHGG in utero alone is capable of generating effector Ts whose activity is detectable for up to 3 mo after birth, as well as long-lived memory Ts which can be reactivated later in life by immunogen challenge.

Discussion

The role of active suppression by T cells in the induction and maintenance of tolerance to T-dependent antigens remains controversial. In the well-characterized

HGG system, T cell-mediated suppression is demonstrable in adoptive transfer for ~30–40 d after tolerance induction (14–18). By contrast, the duration of complete tolerance in the intact animal is of the order of 100 d, and partial unresponsiveness may be observed for >150 d after a single injection of high-dose dHGG (26). Such differences in kinetics have hitherto provided the most cogent argument against the concept of a causal relationship between suppression and tolerance, in this (15) as well as other (27, 28) experimental models. However, the apparent discrepancy can now be satisfactorily resolved in the light of evidence presented here demonstrating that Ts, like other T cell subsets, may exist in two independent physiological states, i.e., as effector cells and memory cells. Thus, after tolerance induction short-lived effector Ts responsible for mediating transient primary suppression were generated, as well as long-lived memory Ts. The latter persisted after primary suppression had waned, but their functional activity only became apparent after secondary antigenic stimulation.

The phenomenon of memory for suppression was first observed in HGG-primed animals (24), and its analysis will be described in detail elsewhere.[2] Briefly, primary carrier-specific suppression was detected 4–5 d after immunization with aHGG and reached a maximum between days 7 and 14, after which suppression waned as helper function became dominant. Subsequent antigenic challenge with dHGG (to restimulate Ts selectively) or with soluble HGG resulted in the appearance of a classical secondary suppressor response, indicating the presence of memory Ts cells in the primed animals. These were shown to be antigen specific, and the phenotype of the reactivated effector Ts was found to be the same as for those mediating primary suppression, i.e. $Ly-1^{-}23^{+}$, $Thy-1^{+}$ Ia^{+}. Since an anamnestic response could be stimulated at least 9 mo after primary immunization it was concluded that the putative memory cells were long-lived.

The experiments described in this paper were designed to determine whether Ts memory could be demonstrated in HGG-tolerant animals. It was reasoned that if such cells were indeed present after primary suppression had waned, and had a life span commensurate with the duration of tolerance, then they might be implicated in maintenance of the unresponsive state. Initially, therefore, animals rendered tolerant with 3 mg dHGG were restimulated with the same dose of deaggregated antigen at various times after primary suppression had subsided. Secondary suppression was assayed in the standard adoptive transfer system and compared with primary suppression induced in parallel in virgin donors. At both 60 and 135 d after tolerance induction suppression could be recalled by a secondary stimulus with dHGG (Fig. 2), and in each case there was significant augmentation of suppressive activity by comparison with the primary effect, suggesting that memory Ts were indeed present in such animals for the full duration of the unresponsive state.

The continued existence (or "maintenance") of a tolerant state is conventionally assessed by the response of the intact animal to a standard immunogenic stimulus. Consequently, before memory Ts could be assigned an etiological role in the maintenance of unresponsiveness to HGG, it was necessary to determine whether they could be reactivated effectively by challenge of the tolerant animal with antigen in immunogenic form. That this was indeed the case was first suggested by the observation of augmented secondary suppression appearing after aHGG challenge of 70-d-tolerant mice, compared with virgin animals given the same challenge dose simulta-

neously (Fig. 3). On further analysis the secondary effect displayed all the classical features of an anamnestic response; thus, its onset was accelerated, its peak heightened, and its duration greatly prolonged by comparison with primary suppression (Figs. 4 and 5). This was exactly analogous to the secondary suppressive response observed in HGG-primed animals (24),[2] and can be attributed to the presence of Ts memory cells in both situations.

On the basis of these data it is possible to construct a working hypothesis concerning the role of Ts in adult tolerance to HGG. Before outlining this it is important to draw a clear distinction between induction and maintenance of the state of unresponsiveness. Inductive events are defined as those that follow the administration of tolerogenic antigen and result in (a) failure to mount a primary immune response to the tolerogen, and (b) establishment of an altered homeostatic state responsible for subsequent specific unresponsiveness. Maintenance of the established tolerant state, on the other hand, involves the specific failure to mount an immune response upon subsequent challenge with antigen in otherwise immunogenic form. In the absence of the usual hallmarks of immunity, tolerance is thus a latent state, the presence of which can only be determined by this failure to respond to challenge.

In the hypotheses proposed here, poor uptake and processing of deaggregated antigen by macrophages (29) may be regarded as setting the stage for the lack of a primary immune response. Inadequate antigen presentation to Th and B cells in the first instance results in the failure to trigger adequate numbers of precursors; those that do happen to be activated are then inhibited from generating an immune response by the appearance of Ts effector cells, the induction of which is known to be macrophage independent (30). Primary suppression may thus be viewed as playing a contributory, though not necessarily essential role in the first stage of tolerogenesis.

More important, however, are those inductive events responsible for generating a prolonged state of specific unresponsiveness. It is proposed here that the central event in this process is the generation of long-lived Ts memory cells, which, in the absence of significant Th memory induction, lead to establishment of a new, quasi-stable state of homeostatic balance. In the absence of antigenic stimulation, such Ts memory cells remain functionally latent, and hence are not detected in the usual mixing experiments designed to assay for suppressor activity. However, immunogen challenge of the tolerant animal leads to reactivation of these cells and the development of manifest suppressive activity. The anamnestic nature of this secondary response results in the accelerated appearance and heightened activity of Ts effector cells, which can then prevent the development of a normal immune response. The tolerant state is thus maintained.

Spontaneous recovery from tolerance is assumed to occur once the number of Ts memory cells has diminished through attrition to the point where their activation is no longer sufficient to prevent the triggering of Th and B cells by an immunogen challenge. A period of hyporesponsiveness preceding full recovery would thus be expected, and this has indeed been observed in the HGG system (26).

The hypothesis outlined above provides a theoretical framework within which it is now possible to comprehend many of the diverse phenomena and apparent inconsistencies associated with adult tolerance. For example, in the HGG system a number of lines of evidence have been interpreted as demonstrating a dissociation between suppression and tolerance, casting considerable doubt over the notion that Ts play a

significant role in the induction and maintenance of unresponsiveness. Thus, certain batches of antigen appear to be capable of inducing tolerance but not suppression (17). Furthermore, it has been shown that the degree of suppression observed in adoptive transfer does not correlate with the duration of unresponsiveness (17), and that colchicine is capable of inhibiting Ts induction without preventing the establishment of unresponsiveness (18). If, as proposed here, a central role is assigned to Ts memory cells, these data can be interpreted as indicating a dissociation not between suppression and tolerance, but between the primary appearance of Ts effector cells and the induction of long-lived Ts memory cells. That memory and effector cell function can indeed be dissociated was directly demonstrated in the present study after the administration of low doses of dHGG. A single dose of 100 µg dHGG was insufficient to generate detectable primary suppression; however an anamnestic secondary Ts response could be elicited by aHGG challenge after a single injection of as little as 1-10 µg of deaggregated antigen, indicating that the latter had been effective in inducing Ts memory. Thus, it would be predicted that in tolerant states not associated with significant primary suppression (17, 28, 31) it should nevertheless be possible to demonstrate the presence of Ts memory cells capable of abrogating the response to immunogen challenge. Experiments are in progress to test this prediction. Interestingly, a similar phenomenon involving the dissociation of effector and memory T cell functions has been described for T cell help (32). In those experiments it was possible to induce good Th memory in the absence of significant primary helper activity by immunizing with very small doses of antigen.

A number of other phenomena associated with the tolerant state can also be reinterpreted in the light of the hypothesis put forward here. For example, prolongation of the tolerant state by immunogen challenge was reported by Smith two decades ago (33), and has been observed by a number of investigators since then (34–39). In the studies of Bell et al. (39), it was shown that not only did challenge inhibit escape from tolerance, but such animals remained hyporesponsive for at least a further 18 mo. Such observations are difficult to account for within the framework of conventional clonal deletion hypotheses of immunological tolerance, but can be readily explained in terms of boosting of Ts memory by immunogen challenge.

Another interesting phenomenon, seen during spontaneous recovery from tolerance, is the secretion of low affinity antibody in response to challenge with immunogen (40). Ts effector cells are known to be capable of selective inhibition of high-affinity antibody production, with or without alteration in magnitude of the response (41–43). It is, therefore, to be expected that during recovery from tolerance the animal will generate sufficient Ts effector cells to alter the affinity profile of the ensuing antibody response without its complete inhibition.

The question whether Ts memory represents the only mechanism of tolerogenesis, or whether individual Th and B cells can be deleted or inactivated independently has not been addressed in the present study. It is, however, known from previously published experiments (19) that thoracic duct lymph from HGG-tolerant mice does in fact contain functional Th memory cells. Furthermore, although evidence for direct inactivation of B cells by tolerogen has been presented in many studies (44–50), it is difficult to be certain whether Ts have been adequately excluded in every case, particularly since in our hands these cells are difficult to deplete exhaustively with anti-Thy-1 and complement (19). A case in point is the induction of unresponsiveness

to HGG in nude mice using an immunizing regimen (51), the implication being that B cells may be directly inactivated in the absence of a second signal. Nude mice are now known to manifest certain T cell functions, including specific suppression (52), and it has been possible to demonstrate the induction of suppressor memory in athymic mice with aHGG in our own laboratory (B. Fazekas de St. Groth, R. H. Loblay, and A. Basten, unpublished observation).

The above experiments provide reasonable evidence for a role for memory and effector Ts in tolerance to foreign antigens during adult life. It is not, however, necessarily valid to extrapolate from these conclusions to the physiological events surrounding self-tolerance, where precursor cells are exposed to antigen during development of the lymphoid system. In an attempt to circumvent these problems and render the experimental model more relevant, animals were exposed to dHGG during fetal development via the transplacental route. After birth they were challenged at various times to determine their responsiveness to HGG. Parallel experiments were performed to test for the presence of specific effector and/or memory Ts. Mice exposed to dHGG in utero were completely tolerant to HGG for the 1st mo after birth. Partial responsiveness began to appear in the intact animals between 6 and 8 wk of age, and by week 9 the level of responsiveness reached that of controls. When tested in adoptive transfer, spleen cells from young animals exhibited marked non-specific suppression and it was only once this had subsided at about 6 wk of age that primary antigen-specific Ts activity was unmasked; the latter persisted until ~10-12 wk of age and then waned (Fig. 8). Memory Ts, however, were demonstrable from week 6 onwards, throughout the entire period of unresponsiveness and beyond, still being detectable up to 6 mo later (Fig. 9). The possibility that these cells might have been induced postnatally by exposure to antigen through suckling was excluded by cross-fostering.

The experiments described here indicate that exposure of animals to antigen during a very early stage of development of the immune system can lead to generation of long-lived antigen-specific Ts memory cells which persist well into adult life. This is in contrast to Th memory, which cannot be effectively established until the potent nonspecific suppression present for the first few weeks of extrauterine life has subsided (53).

A similar model of in utero tolerance induction has been reported by Waters et al. (27). In those experiments it was found that the animals were unresponsive until ~12 wk of age, but primary suppression was detected only in the recovery phase. The authors concluded that Ts were therefore unlikely to be involved in the generation of tolerance in this model, and favored clonal deletion as the probable mechanism. However, nonspecific suppression was not assayed in their study, and as shown here this could have been responsible for the masking of specific suppression during the period of postnatal unresponsiveness. The present study suggests that two factors may be responsible for tolerance after exposure to antigen during fetal life in the mouse. In neonatal and early adult life the combined presence of nonspecific Ts (53) and antigen-specific Ts results in a period of complete unresponsiveness; as nonspecific suppression wanes, this developes into a state of partial unresponsiveness maintained by memory Ts alone. The ability to extend the duration of complete tolerance by intermittent injections of dHGG in such animals (27) can thus be attributed to boosting of the memory Ts population.

By analogy with the above model, it may be concluded that memory Ts could be responsible for maintenance of self-tolerance. Exposure to very small quantities of self-antigen throughout life would be capable of continually replenishing the pool of memory cells without necessarily generating any detectable Ts effector activity, as shown here with HGG.

Summary

The transient presence of suppressor T cell (Ts) activity in high-dose tolerance to human gamma globulin (HGG), and its (apparent) absence in low-dose tolerance, have been advanced as strong evidence against the concept that Ts play an important role in maintenance of immunological unresponsiveness. To analyze this question, CBA mice were exposed to high or low doses of deaggregated HGG (dHGG) and later challenged with HGG in immunogenic form (aHGG); their capacity to mount a primary or secondary suppressive response was assessed in an adoptive hapten-carrier system. Primary suppression reached a maximum 7 d after high-dose tolerance induction and gradually waned thereafter, being no longer detectable by day 30–35. Subsequent challenge of tolerant mice with aHGG, however, led to a rapid reactivation of suppression that bore the hallmarks of an anamnestic secondary response, and this effect was still demonstrable 135 d after tolerance induction. It was also shown that a single low dose of dHGG was capable of generating memory for suppression despite the absence of detectable primary suppression, indicating that the latter is not a prerequisite for induction of memory cells. The results were interpreted as indicating that tolerance, like immunity, is a manifestation of specific immunological memory.

If tolerance to self-antigens is maintained by a similar mechanism, it would be expected that memory Ts could be induced during the early stages of fetal development. Mice were therefore exposed to tolerogen in utero by injection of their mothers with dHGG at day 7 of gestation, and were assessed at various times after birth for the capacity to exhibit primary or secondary suppression in adoptive transfer. Nonspecific suppression masked any specific effects during the first 5 wk of life. Antigen-specific, primary suppression was demonstrable subsequently until 10–12 wk of age, and if the animals were challenged with aHGG before transfer an anamnestic secondary suppressive response could be elicited up to 6 mo of age. These observations are consistent with the notion that memory Ts may play an important role in the maintenance of self-tolerance.

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