Immunoregulatory Abnormalities in Autoimmune Thyroid Diseases

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Abstract

We have investigated the ability of lymphocytes from normal subjects and patients with autoimmune thyroid diseases to respond to a thyroidal antigen (human thyroglobulin, hTG) and a non-thyroidal antigen (Keyhole limpet hemocyanin, KLH) in vitro, using a hapten (trinitrophenol, TNP)-carrier system. This system is based on the concept that the T helper cells which respond to hTG or KLH should stimulate anti-TNP antibody producing B cells in the presence of TNP conjugated hTG (TNP-hTG) or KLH (TNP-KLH). After 5 or 6 days of culture of peripheral blood mononuclear cells with pokeweed mitogen (PWM), PWM and TNP-hTG, or PWM and TNP-KLH, IgM anti-TNP and IgM anti-sheep red blood cell (SRBC) plaque forming cells (PFC) were enumerated. The results showed that (1) in normal controls, hTG caused only suppression in both TNP and SRBC response, and KLH caused dose-related enhancement and suppression in TNP response without a change in SRBC response, and (2) in patients, both hTG and KLH resulted in dose-related enhancement in TNP response without a change in SRBC response. These data suggest that patients with autoimmune thyroid diseases have regulatory cell abnormalities confined to a thyroid antigen.

In patients with autoimmune thyroid diseases the occurrence of HLA-DR3 and DR5 antigens is increased (Farid and Bear, 1981). It is believed that these HLA-DR antigens are associated with genetically determined abnormal immune responses. Many of the patients have a positive family history of these diseases, and children of patients with Graves' disease have a high incidence of auto-antibodies to the thyroid (Carey, et al., 1980). The basic pathogenesis of autoimmune thyroid diseases could be that patients have a genetic predisposition to respond to thyroid antigens more readily than normals.

Previous studies on the regulation of in vitro anti-human thyroglobulin (hTG) antibody production have suggested both B cell and T cell abnormalities (Beall and Kruger, 1979; McLachlan et al., 1980; Weiss et al., 1982; Noma et al., 1982). However in some of these studies allogeneic lymphocytes were mixed (Beall and Kruger, 1979;
McLachlan et al., 1980; Noma et al., 1982), and possible allogeneic effects (Golding and Rittenberg, 1982; Callard and Smith, 1981; Brenner and Munro, 1981) were not clearly excluded. In other studies nonspecific manipulation of suppressor T cells was employed (Beall and Kruger, 1979; Weiss et al., 1982).

In this study we intended to investigate possible regulatory abnormalities in the response of lymphocytes to hTG, a thyroid specific antigen, and to keyhole limpet hemocyanin (KLH), a nonthyroid specific antigen. When we study possible T cell abnormalities altering antibody production, differences in the frequency of hTG specific B cells between patients and normal subjects could cause a problem. To avoid this problem we used hapten (TNP) conjugates of hTG and KLH, and measured anti-TNP antibody producing B cells. These TNP-specific B cells should be of similar frequency in patients and normal populations. In this system, it has been documented that carrier (hTG or KLH) specific T helper cells or factors can induce hapten (TNP) specific response in B cells, provided the hapten (TNP) is coupled to the original carrier antigen (hTG or KLH) (Dosch et al., 1982; Heijnen et al., 1981). Therefore carrier protein specific immune response can be detected by the number of TNP plaques.

### Materials and Methods

1. **Subjects**

Thirty patients with autoimmune thyroid disease (24 with Graves', 6 with Hashimoto's disease) and 22 normal controls were tested. In the patient group, 10 were TGHA positive (> 1:40) and 20 were negative. Among patients with Graves' disease, 3 were toxic without treatment, 3 were euthyroid on PTU, and 18 were euthyroid and in remission for more than one year after various treatments. All the patients with Hashimoto's disease had high TGHA titers (>1:6,400) and were hypothyroid at the time of diagnosis, but were euthyroid when this study was done after replacement with thyroid hormone. Data about age and sex are shown in Table 1.

2. **Antigens**

hTG was purified from the tissue of a patient

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| Table 1. Anti-TNP maximal response to TNP-hTG and TNP-KLH. |
|-----------------|-----------------|-----------------|-----------------|
|                 | Culture conditions |
|                  | Age n | Sex F/M | PWM alone n | PWM + TNP-hTG n | PWM + TNP-KLH n |
| Groups          | mean (S.D.) |         | mean (S.E.) | mean (S.E.) | mean (S.E.) |
| Normal control  | 29.7 (6.6)   | 13/9    | 22 24.4 (5.2) | 12 24.2 (6.0) | 18 43.1$^2$(7.8) |
| Patients, total | 45.2 (11.8)  | 24/6    | 30  7.0$^1$ (1.7) | 29 30.9$^1$(5.1) | 23 39.3$^1$(9.7) |
|                |               |         |               |               |               |
| Graves' disease | 34.0 (6.0)   | 6/0     | 6 12.3 (4.3)  | 6 25.3 (9.6)  | 5 24.4 (7.4)  |
| tox or on PTU   |               |         |               |               |               |
| Graves' disease | 46.2 (10.9)  | 12/6    | 18 5.7$^1$(2.2) | 17 28.1$^1$(7.2) | 12 38.8$^1$(12.0) |
| in remission    |               |         |               |               |               |
| Hashimoto's     | 53.5 (10.4)  | 6/0     | 6 5.5$^1$(2.3) | 6 44.5$^1$(7.9) | 6 52.8 (26.4) |
| disease         |               |         |               |               |               |
| TGHA (+)        | 49.6 (11.5)  | 7/3     | 10 4.3$^2$(1.2) | 10 35.0$^2$(6.3) | 8 41.6$^2$(19.8) |
| TGHA (-)        | 43.1 (11.3)  | 17/3    | 20 8.3$^2$(1.9) | 19 28.8$^2$(7.0) | 15 38.1$^2$(9.7) |

On comparison to the value of “PWM alone” in the same group, $^1$ p<0.005 and $^2$ p<0.05.

On comparison to the value of normal controls in the same culture condition, $^1$ p<0.01 and $^2$ p<0.05.
with Graves' disease by salting-out with ammonium sulfate followed by Sephadex G-200 column chromatography (Ui and Tarutani, 1961; Salvatore et al. 1964). Conjugation of TNP to hTG was carried out according to Rittenberg and Amkraut (1966). TNP-KLH was kindly donated by Dr. J. Quintans (Larabida—University of Chicago Research Institute). Molar ratios of TNP-residues of protein in the conjugates were 98 for TNP-hTG and 40 for TNP-KLH (per 10^6 daltons).

3. Cell Cultures
Peripheral blood mononuclear cells were separated by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, NY), and resuspended in the culture medium, which contained 10\% heat inactivated human serum, 5\times10^{-5} M 2-mercaptoethanol and 1\% penicillin-streptomycin-amphotericine B cocktail (GIBCO) in RPMI 1640 with glutamine. The cells were cultured at a concentration of 3-5\times10^6/ml in duplicate tubes (17\times100 mm. Falcon, Cockeysville, MD) with 100\% humidity in a 5\% CO2 incubator. Pokeweed mitogen (PWM, GIBCO) was added at a final dilution of 1:500. TNP-hTG or TNP-KLH was also added at final concentrations of 0, 1, 10, 100, and 1,000 ng/ml.

4. Plaque Forming Assay (PFA)
TNP-sheep red blood cells (TNP-SRBC) were prepared according to Rittenbreg and Pratt (1969). At the end of 5 or 6 days of culture, cells from duplicate tubes were combined and washed twice with Ca and Mg free Dulbecco’s phosphate buffered saline, and resuspended in 0.6-1 ml of RPMI 1640 medium. Cells were used for anti-TNP PFA and anti-SRBC PFA. 0.1 ml of lymphocyte suspension, 0.05 ml of 6\% TNP-SRBC or SRBC suspension and 0.025 ml of 1:2 diluted guinea pig complement were mixed and poured into a Cunningham slide (Cunningham and Szenberg, 1968). After 1-2 hr of incubation at 37\°C, plaques were counted under a dissecting microscope. Anti-TNP response was expressed as the number of anti-TNP plaque forming cells (TNP-PFC) per 10^7 initial cells, after subtraction of background of anti-SRBC PFC (SRBC-PFC).

5. Statistics
The Wilcoxon signed rank test for a paired experiment was used in the dose response study.

Student t-test was used in the maximal response study.

Results

1. Necessity of PWM and response to PWM alone
We first tried to develop antigen-specific antibody responses without adding nonspecific mitogens, but we could not detect any anti-TNP or anti-SRBC response without adding PWM. We then added PWM in our culture, at a 1:500 dilution. In preliminary experiments 1:100 to 1:1,000 dilution resulted in about the same number of PFC. As shown in Table 1 and Figure 4, anti-TNP response in patients to PWM alone was lower than that in normal controls, whereas anti-SRBC response to PWM alone was the same as that of normal controls. In other words, patients' PBL seemed to have some specific difficulties in producing TNP-PFC when they were stimulated with PWM alone.

2. Time course of the development of TNP-PFC and SRBC-PFC (Fig. 1)
As shown in Fig. 1. TNP-PFC began to appear on day 5, remaining at the same level on day 6, and disappeared after day 7. Background SRBC-PFC also appeared on day 5, and increased to the maximum on day 7 and decreased thereafter. These two types of PFC have a different chronological course of development. In order to get specific anti-TNP PFC, we carried out PFA on day 5 or 6.

3. Dose related anti-TNP response to TNP-hTG (Fig. 2)
When the lymphocytes were stimulated by TNP-hTG and PWM, the number of TNP-PFC in normal controls was reduced at higher concentrations of TNP-hTG (Fig. 2-A). Since there was no difference in cell viability among tubes, the reduction of the
number PFC in normal subjects was not due to a toxic effect of TNP-hTG, but seemed to be related with the induction of suppressor T cells. On the other hand, the number of TNP-PFC in patients was not reduced at higher antigen concentrations and, furthermore, was enhanced at two concentrations (1 and 100 ng/ml) (Fig. 2-B). We then divided the patient group into subgroups on the basis of clinical diagnosis (Fig. 2-C, D), or the presence or absence of anti-hTG autoantibody (TGHA) (Fig. 2-E, F). In patients with Graves’ disease who had been in remission for more than one year, there was no reduction in PFC at higher concentration of TNP-hTG (Fig. 2-C), which suggested that the abnormalities were not related to the thyroid function and that they might be the primary abnormalities in those patients. The enhancement of the response was more demonstrable in patients with Hashimoto’s disease (Fig. 2-D) or with positive TGHA (Fig. 2-F).

Comparison of the absolute numbers of PFC at each antigen concentration in normal controls and the patient group did not show a difference.

4. Dose related anti-TNP response to TNP-KLH (Fig. 3)

When the cells were stimulated by TNP-KLH and PWM, the number of TNP-PFC in normal controls did not show a statistically significant change according to the concentrations of TNP-KLH (Fig. 3-A), although there was a tendency to increase at lower concentrations and to decrease at higher concentrations. The number of TNP-PFC in patients was lower in relation to PWM alone than normal controls, as stated previously, but it rose to the same levels as the normal controls with the addition of small amount of TNP-KLH, and was suppressed at higher concentration of TNP-KLH to the same extent as the normal controls, resulting in a typical, bellshaped dose related response curve. This pattern of enhanced response was also seen in all subgroups of patients (Fig. 3-C, D, E, F).

TNP-KLH did not cause any decrease
5. Background SRBC-PFC (Fig. 4)

In normal controls, the SRBC-PFC numbers were suppressed when TNP-hTG was added, but they were not influenced by TNP-KLH. In patients, they were not influenced by TNP-hTG or TNP-KLH. There was no difference between the number of SRBC-PFC in normal controls and that in the patient group at each concentration of TNP-hTG or TNP-KLH.

6. Maximal anti-TNP response (Table 1)

Because the optimal concentration of the antigens varied according to the individual, we analyzed the maximal response in each subject. In the normal controls, the maximum number of TNP-PFC resulting from stimulation with TNP-KLH was significantly higher than that of the control...
cultures (PWM alone), whereas those with TNP-hTG were not increased at all. On the other hand, patient PBL showed increased response to both antigens.

Compared to the value for normal controls, the response to TNP-hTG in patients with Hashimoto's disease showed a significant increase, although the response to both antigens in the other groups of patients and patients as a whole did not show a difference. In the patient group, anti-TNP response in the cultures with PWM alone was smaller than that in normal controls. Therefore, the major differences between normal controls and patients were that the patients showed a poor response to PWM alone and a clear and enhanced response to hTG.

Fig. 3. IgM anti-TNP response to TNP-KLH. A-F represent the same groups as in Fig. 2. A: n=17, B: n=23, C: n=12, D: n=6, E: n=15, F: n=8. Mean and standard errors are shown. Significant difference (p < 0.005) is shown by the triangles.
Fig. 4. Antigen dose related background IgM anti-SRBC response. Mean and standard errors are shown. There are no significant differences between patient and control samples or among various concentrations of antigens.

*1 On comparison with PWM alone, p<0.05

Discussion

Several groups have reported on in vitro anti-TNP and -DNP antibody production in humans using TNP or DNP conjugates of different protein antigens or polyacrylamide beads (Dosch et al., 1982; Heijnen et al., 1981; Watanabe et al., 1974; Delfraissy et al., 1977; Morimoto et al., 1981). T cells are required in this anti-hapten antibody response, even if the TNP conjugate is in insoluble form. Furthermore, carrier antigen specific T helper cells and T suppressor cells or their factors (induced in vitro by a low and high dose of antigens, respectively) were shown to regulate the anti-hapten antibody response, provided the hapten is coupled to the original carrier antigens (Dosch et al., 1982; Heijnen et al., 1981). Therefore, these systems allow us to investigate regulatory cell (T cells and macrophages) abnormalities in relation to protein antigens through the number of anti-TNP plaques, without concern for the B cell phenomenon related to that antigen. This theoretically has a great advantage in an investigation of regulatory cell abnormalities in autoimmune diseases, in which both B cell and T cell abnormalities have been implicated.

Although this kind of study should be carried out without nonspecific mitogenic stimulants, it was necessary for us to add a low dose of PWM because we could not demonstrate any anti-TNP response without PWM. However, the use of PWM does not prevent our studying the regulatory cell phenomena in relation to carrier antigens, because PWM is primarily a T cell mitogen (Keightley et al., 1976) and has a synergistic effect with an antigen (Khansaril et al., 1983).

In the present study, the optimal concentration of TNP conjugate and the number of PFC varied very much from person to person. We analyzed our data in two ways, by the dose response curve (Fig. 2, 3, 4) and by maximal response in the cultures.
with TNP conjugates (Table 1).

The first finding was that the TNP response in patients to PWM alone was lower than that in normal controls (Table 1). The general responsiveness of T cells to PWM did not seem to differ between the two groups since other investigators reported that lymphocytes from patients with autoimmune thyroid diseases produced the same magnitude of IgG by stimulation with PWM as normals (McLachlan et al., 1980; Weiss and Davies, 1982), and also, our data about background SRBC-PFC production by the stimulation with PWM alone were similar for both groups. The number in the TNP-specific B cell population also seemed similar since our data for maximal response with antigens were the same for both groups. Therefore, in patients with autoimmune thyroid diseases, TNP-specific helper factor production by PWM alone or TNP-specific B cell responsiveness to that factor might be deficient. However, both possibilities remained to be investigated further.

The second finding was the difference between the response to hTG in normal controls and patients. In normal controls, hTG caused suppression of both TNP and SRBC response, suggesting the induction of non-specific suppressor T cells by hTG. On the other hand, in patients, hTG caused the enhancement of TNP response and no change in SRBC response, suggesting the presence of hTG-specific helper T cells and deficiency in non-specific suppressor T cells. The presence of hTG-specific helper T cells might be related to a lack of specific suppressor T cells. No matter how the mechanism is, these results demonstrated the presence of regulatory cell abnormalities in patients with autoimmune thyroid diseases. The results that maximal response to hTG in patients with Hashimoto’s disease was the highest and significantly higher than normal controls well reflects the clinical observation of their high TGHA titers in the blood. Moreover, the finding that patients with Graves’ disease in remission for more than a year showed the same kind of abnormalities suggests a pathogenetic role of those regulatory cell abnormalities and a genetic predisposition to respond to thyroid antigen more readily than normals.

The third finding was a similar response to TNP-KLH in both normal controls and patients. Both groups showed some enhancement in TNP-PFC response at lower antigen concentrations and suppression at higher concentrations. The analysis of maximal TNP-PFC response to KLH showed more definite response in both groups. However, KLH did not cause any change in SRBC-PFC response in either group. KLH therefore seemed to induce KLH-specific suppressor T cells in both groups. These results suggest that there is no abnormality in the immunoregulation of KLH in patients with autoimmune thyroid diseases.

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**References**


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