PHYSIOLOGY OF IgD

IV. Enhancement of Antibody Production in Mice Bearing IgD-secreting Plasmacytomas

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It is known that mice bearing plasmacytomas have an impaired immune response to both thymus-dependent (TD)[1] and thymus-independent (TI) antigens (1–4). This effect is to a large extent unrelated to paraprotein production (5) and is greatest on the primary response, although decreases in the secondary response are frequent (1).

Recently two IgD-producing plasmacytomas, called TEPC-1017 and -1033, have been described (6), but no reports on their ability to affect immune responses have appeared. In view of the presence of IgD on the surface of the majority of B cells, in spite of its extremely low representation in serum, many hypotheses about the role of IgD as a differentiation (7, 8) or proliferation (9) trigger of B cells have been put forth. The presence of IgD on B cells during TD immune responses in vitro appears to be particularly important (10).

On the other hand, subpopulations of T cells are known to have receptors for Fc portions of a variety of Ig classes, including IgM (11), IgG (12), IgA (13), and IgE (14). Such cells have been shown to modulate immune responses both upward and downward (15–17). No T cell populations with receptors for IgD have been described. If such cells exist and can modulate the immune response, then it might be expected that IgD-secreting plasmacytomas would indirectly influence immune responses, since they would cause a marked change in serum IgD levels. In the present study, we show that TD immune responses of TEPC-1017- and TEPC-1033-bearing mice are greatly enhanced and that treatment with IgD-containing ascites fluid can mimic this effect of IgD-secreting plasmacytomas on the host.

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[1] *Abbreviations used in this paper:* BA, *Brucella abortus*; DNP, dinitrophenyl; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; PFC, plaque-forming cell; SE, sheep erythrocyte; TD, thymus dependent; TI, thymus independent; TNP, trinitrophenyl.

Materials and Methods

Mice. BALB/c mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. Athymic BALB/c mice were supplied by the Division of Cancer Treatment Animal Program, National Cancer Institute, Bethesda, MD.

Antigens and Adjuvants. Sheep erythrocytes (SE) were purchased from Colorado Serum Co., Denver CO; keyhole limpet hemocyanin (KLH) from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY; Ficoll, 40,000 mol wt, from Pharmacia Fine Chemicals, Piscataway,NJ; and trinitrophenyl sulfonic acid from Sigma Chemical Co., St. Louis, MO. *Brucella abortus* (BA) was provided by the U. S. Department of Agriculture, Ames, IA. Trinitrophenyl (TNP) and dinitrophenyl (DNP) conjugates were prepared as described by Little and Eisen (18), except for TNP-Ficoll, which was prepared according to the method of Mishell and Dutton (19). *Bordetella pertussis* (Massachusetts Public Health Biology Laboratories) and *Escherichia coli* endotoxin (lipopolysaccharide [LPS]) (Difco Laboratories, Detroit, MI) were used as adjuvants in some experiments.

Immunization. Mice were injected intravenously at 6–8 wk of age (day 0) with either 10 μg TNP-Ficoll, 100 μg TNP-KLH, 100 μg DNP-ovalbumin (DNP-OVA), or 80 μg TNP-BA. Primary responses to TNP-Ficoll and TNP-BA were determined on day 4, and to TNP-KLH and DNP-OVA on day 5 or 7 after immunization. For secondary responses, mice injected with TNP-BA and TNP-KLH were boosted 14 d later, and their responses were determined 5 d after boosting. Some mice received 10 μg TNP-KLH in the front footpads.

Plasmacytomas. Plasmacytomas were serially transplanted by intraperitoneal injection of 10^6 cells in BALB/c mice. Ascites cells and fluids were collected 2 wk after inoculation of tumor cells. Subcutaneously growing tumors were obtained by the injection of minced tumor pieces. To study the effect of these tumors on the immune response, we injected mice bearing subcutaneous tumors with a primary dose of antigen 2–3 wk after tumor injection. Serum and ascites fluid were examined for the presence of IgD by double diffusion in agar (20) using goat anti-mouse δ prepared by immunization of a goat with myeloma protein from TEPC-1033. The antiserum was absorbed in sequence with Sepharose 4B coupled to mouse Ig, MOPC-104E (μ, λ), UPC-10 (γ2a, κ) and Y 5606 (γ3, λ). The absorbed antiserum no longer reacted with normal mouse serum. The IgG fraction of this antiserum, prepared by chromatography on DEAE cellulose, was conjugated to fluorescein or rhodamine and used to stain viable mouse spleen and lymph node cells for surface IgD. Double staining with goat anti-mouse Fab conjugated to a different fluorochrome than that used for the anti-IgD was used to determine the percentage of IgD-bearing B cells. Surface staining was examined as described previously (21).

Purification of IgD. TEPC-1017 ascites was passed over a 2-g column of goat anti-mouse IgD-coupled Sepharose 4B (Pharmacia Fine Chemicals). After extensive washing with phosphate-buffered saline, the IgD bound to the column was eluted with 4.0 M $MgCl_2$. This eluate was dialyzed and reconstituted to the same volume as the original ascites fluid. It formed a precipitin band with anti-mouse IgD upon double diffusion in agar.

Plaque-forming Cell (PFC) Assay. Anti-SE and anti-TNP-PFC were assayed by the slide modification of the technique of Jerne et al. (22). TNP-SE were prepared using the method described by Rittenberg and Pratt (23). IgG-producing cells were developed with rabbit anti-mouse Ig in the complement and goat anti-μ in the agar (24).

Results

The plasmacytomas became palpable 10–14 d after subcutaneous injection in ~90% of injected mice. No IgD could be detected by double diffusion in agar in serum from normal mice. IgD became detectable in the serum of tumor-bearing mice in ~2 wk and remained high until the mice became moribund 6–8 wk after tumor injection. Sera taken ~4 wk after the injection of tumor reacted at dilutions ranging from 1:16 to 1:32. In mice that received 10^6 tumor cells i.p., the ascites

fluid collected 10–14 d later contained sufficient IgD to be detectable up to dilutions of 1:32. After ~5–6 wk, small tumor nodules could be seen in the spleen. Mice showing such evidence of splenic plasmacytoma growth were not used for the analysis of PFC. Expressed as the percentage of body weight, spleens from tumor-bearing mice were approximately twice as large as spleens in control mice. The percentages of B cells showing membrane IgD were similar in spleen and lymph node cell suspensions from IgD myeloma-bearing and control mice. The values in all cases fell between 80 and 95%.

The effects of TEPC-1017 and -1033 on the splenic PFC responses after primary or secondary intravenous injections of TNP-KLH are presented in Fig. 1. Both tumors enhanced the primary 19S and 7S responses three to fourfold, whereas the secondary response induced 2 wk after priming was enhanced three to fourfold by TEPC-1033 and as much as four to fivefold by TEPC-1017. The primary response to 10 μg TNP-KLH injected into the front footpad was measured in the draining brachial lymph node. These responses were very low in control mice (110 PFC/node), but again increased fourfold in TEPC-1017-bearing mice (data not shown). Secondary responses induced in the draining lymph node after a 14-d interval were also threefold enhanced for both 19S and 7S PFC in the TEPC-1017-bearing mice. Some mice, which had been injected with minced tumor but failed to develop palpable tumors, were also injected with TNP-KLH 3 wk later. Primary responses in these mice were three to fourfold higher than in control mice (data not shown). It is likely that in these mice there was production of IgD, but not to a level detectable in serum by immunoprecipitation; the serum IgD levels might have been similar to those obtained in mice injected with IgD-containing ascites (see below). We also studied a few mice that had failed to grow palpable tumors after subcutaneous injection with 10^4–10^6 dissociated plasmacytoma cells. These mice showed only approxi-

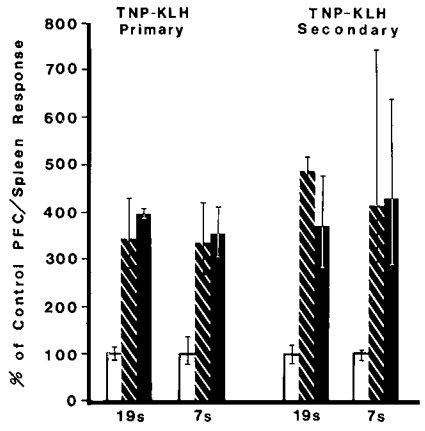

FIGURE 1. Responses of IgD-secreting, myeloma-bearing mice to TNP-KLH. PFC per spleen responses determined on day 5 after a primary intravenous injection of 100 μg TNP-KLH or after a secondary intravenous injection 2 wk later. Responses of tumor-bearing mice are expressed as percentage of control responses, for TEPC-1017 (striped bars) and TEPC-1033 (solid bars). n = 10, 11, and 7 for primary response; 100% values for PFC/spleen were 19,000 (19S) and 7,400 (7S). n = 4, 4, and 3 for secondary response; 100% values were 25,100 (19S) and 57,200 (7S) in one experiment and 3,500 and 15,500 PFC/spleen in the other.

mately twice higher 19S and a slightly increased 7S primary response as compared with control mice, upon immunization 4 wk after tumor cell injection.

The primary 19S and 7S responses of mice injected with 800 μg TNP-BA i.v. 2 wk after receiving transplants of either TEPC-1017 or -1033 were depressed to ~50% of the control levels (data not shown). Primary 19S and 7S responses to 80 μg TNP-BA were enhanced two to threefold in TEPC-1017-bearing mice, but were depressed by TEPC-1033 (Fig. 2). In contrast, both tumors greatly enhanced secondary responses to TNP-BA: three to fourfold for TEPC-1033 and four to sevenfold for TEPC-1017. Since preparation for a secondary response is much more T dependent than the primary response (25), these results suggested that the IgD-producing plasmacytomas caused a preferential increase in TD responses.

Fig. 3 shows the responses of TEPC-1017- and -1033-bearing mice to TNP-Ficoll. It was again noted that TEPC-1017 enhanced both 19S and 7S responses by a factor of 1.5–2 whereas TEPC-1033 only enhanced the 7S response. The effect on responses to two different TNP-Ficoll preparations was comparable.

We compared the effect of TEPC-1017 with that of other agents that enhance immune responses. The results in Table I show that the primary response to intravenously injected DNP-OVA was enhanced to approximately the same level in TEPC-1017-bearing mice as it was in normal mice given 10^9 *B. pertussis* organisms intravenously, simultaneously with the antigen. Responses in TEPC-1017-bearing mice to DNP-OVA and *B. pertussis* were not significantly enhanced over those in control mice injected with antigen and *B. pertussis*. The data in Table II show that the secondary response to intravenously injected DNP-OVA was extremely low and lacked a significant 7S component. Although the 19S PFC were again higher in TEPC-1017-bearing mice, the 7S PFC response

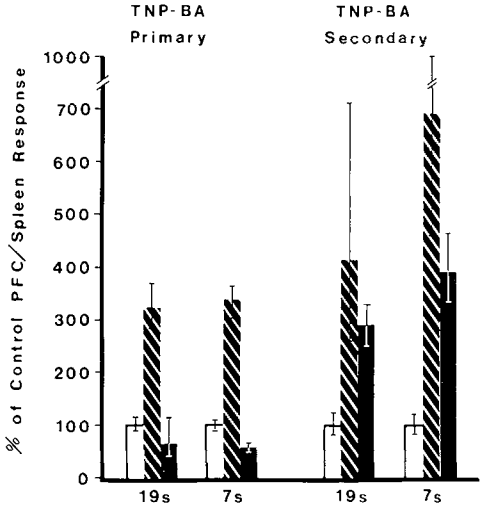

FIGURE 2. Responses of IgD-secreting, myeloma-bearing mice to TNP-BA. PFC per spleen responses determined 4 d after intravenous injection of 80 μg TNP-BA, presented as in Fig. 1. n = 4, 3, and 3 for primary response; 100% values for PFC/spleen were 100,000 (19S) and 41,700 (7S). n = 9, 5, and 3 for secondary response; 100% values were 26,300 (19S) and 57,400 (7S) PFC/spleen.

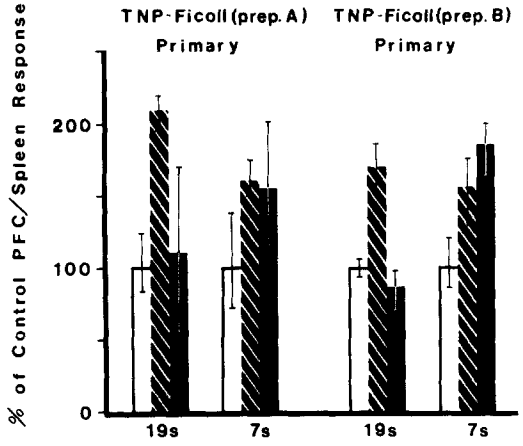

FIGURE 3. Primary responses of IgD-secreting, myeloma-bearing mice to two different preparations of TNP-Ficoll. PFC per spleen were determined on day 4 after intravenous injection of 10 μg TNP-Ficoll, presented as in Fig. 1. (Prep. A) n = 7, 9, and 4; 100% values for PFC/spleen were 338,800 (19S) and 180,800 (7S). (Prep. B) n = 8, 8, and 5; 100% values for PFC/spleen were 240,500 (19S) and 23,000 (7S).

TABLE I

Effect of TEPC-1017 on the Primary Immune Response to DNP-OVA With and Without B. pertussis

B. pertussis injected day 0	TEPC-1017 bearing	Days after antigen	Geometric mean PFC/spleen $\times/\div$ SE (n)*	
			19S	7S
–	–	5	800 $\times/\div$ 1.1	1,000 $\times/\div$ 1.1 (2)
–	+	5	5,000 $\times/\div$ 1.7	2,000 $\times/\div$ 1.4 (3)
+	–	5	6,200 $\times/\div$ 1.2	1,300 $\times/\div$ 1.3 (9)
+	+	5	8,800 $\times/\div$ 1.5	2,000 $\times/\div$ 1.2 (3)
+	–	7	4,400 $\times/\div$ 1.2	835 $\times/\div$ 1.4 (5)
+	+	7	7,900 $\times/\div$ 1.0	1,600 $\times/\div$ 1.2 (3)

* PFC/spleen determined on day 5.

remained insignificant. Mice injected with DNP-OVA plus adjuvants, in contrast, had excellent 19S and 7S responses, which were further enhanced by the presence of growing TEPC-1017. The degree of enhancement caused by the IgD-secreting plasmacytoma was much greater in the mice immunized with DNP-OVA and LPS than in the mice receiving DNP-OVA and *B. pertussis*.

The effect of growing IgD-secreting tumors could be reproduced by pretreatment of the mice with four injections of 1 ml of IgD-containing ascites fluid (Table III). The amount of IgD injected was not sufficient to yield detectable serum IgD in the recipient mice 20 h after a single injection or 1 wk after multiple injections of ascites fluid. The control responses to TNP-KLH in these

TABLE II

Effect of TEPC-1017 on the Secondary Immune Response to DNP-OVA With and Without Adjuvants

Response to:*	TEPC-1017 bearing‡	Geometric mean PFC/ spleen ×÷ SE (*n*)§	
		19S	7S
DNP-OVA + LPS	−	36,600 ×÷ 1.3	53,600 ×÷ 1.3 (3)
	+	161,900 ×÷ 1.3	325,700 ×÷ 1.2 (3)
Percent of control		442%	658%
DNP-OVA + *B. pertussis*	−	50,500 ×÷ 1.3	99,200 ×÷ 1.1 (3)
	+	72,400 ×÷ 1.2	223,900 ×÷ 1.1 (2)
Percent of control		143%	226%
DNP-OVA alone	−	2,900 ×÷ 1.3	500 ×÷ 1.7 (6)
	+	6,100 ×÷ 1.1	300 ×÷ 1.3 (6)
Percent of control		207%	56%

* Interval between primary and secondary intravenous injections of 100 μg DNP-OVA was 10 d.

‡ Primary injection was given 2 wk after injection of TEPC-1017 subcutaneously.

§ PFC per spleen determined 4 d after secondary injection.

experiments are somewhat higher than in the experiments represented in Fig. 1 because a different preparation of TNP-KLH was used. However, the magnitude of the 19S and 7S responses was increased three to fivefold by pretreatment with ascites fluid 1 wk before antigen, while injection of ascites fluid starting 1 d before antigen caused enhancement primarily of the 7S response (Exp. 1, Table III). Ascites fluid prepared with a non–IgD-producing plasmacytoma (MOPC-321 G41, a kappa chain producer) decreased rather than increased the primary response to TNP-KLH. Furthermore, the results in Fig. 4 show that a comparable enhancing effect on the primary IgM response to TNP-KLH is obtained with two different IgD-containing myeloma ascites fluids as well as with the IgD isolated from one of them.

To determine whether the effect of IgD was dependent upon the presence of T cells, experiments on the primary response were repeated with nude (athymic) mice. The results of such an experiment are included in Table III (Exp. 3). They show that pretreatment with ascites, according to the same schedule that caused enhanced responses in normal mice, failed to increase the response of athymic mice.

Discussion

The present results clearly demonstrate that IgD-secreting myelomas enhance both 19S and 7S antibody responses of the host. This is an effect opposite to that described for plasmacytomas that either do not secrete any Ig or secrete an

TABLE III

Effect of IgD-containing Ascites Fluid on Primary Immune Response to TNP-KLH

Exp.	Mice	Ascites fluid* injected	Time of injections‡	Geometric mean PFC/spleen ×÷ SE (*n*)§	
				19S	7S
1	BALB/c	None		16,800 ×÷ 1.4	22,400 ×÷ 1.4 (4)
		TEPC-1017	−8, −7, −6, −5	51,900 ×÷ 1.2	97,200 ×÷ 1.1 (4)
		TEPC-1017	−1, 0, +1, +2	20,800 ×÷ 1.1	84,600 ×÷ 1.2 (4)
2	BALB/c	None		23,400 ×÷ 1.1	26,700 ×÷ 1.2 (10)
		TEPC-1017	−8, −7, −6, −5	86,100 ×÷ 1.1	98,500 ×÷ 1.7 (3)
		MOPC 321G41	−8, −7, −6, −5	14,600 ×÷ 1.1	14,700 ×÷ 1.1 (4)
3	Athymic	None		3,500 ×÷ 1.3	<100 (5)
	BALB/c	TEPC-1017	−8, −7, −6, −5	3,700 ×÷ 1.2	<100 (4)

* Ascites fluid collected 10–14 d after injection of 10^6 tumor cells intraperitoneally.

‡ Time of injection is indicated in days, taking day 0 as the day of intravenous injection of 100 μg TNP-KLH. Mice received four injections of 1 ml; the first and third were part intravenous and part intraperitoneal and the second and fourth were intraperitoneal only.

§ PFC/spleen determined on day +5. Anti-μ added to agar for determination of 7S PFC.

Ig other than IgD (1–5). Since pretreatment with ascites fluid from IgD-secreting myeloma-bearing mice, and with isolated IgD from such a fluid, also causes a marked enhancement of the PFC response to TNP-KLH, this effect appears to be mediated by IgD itself rather than by some other unidentified product of the myeloma cells. It should be noted that, in spite of considerable variability in the magnitude of the responses detected in untreated mice, probably due to the use of different antigen batches, the enhancing effect of IgD on the primary response was reasonably consistent.

The mechanism by which IgD enhances immune responses does not appear to be via a direct effect on B cells. In the first place, the percentage of IgD^+ B cells does not change after pretreatment with IgD. Secondly, treatment with IgD-containing ascites 1 wk before antigen injection causes a greater enhancement of the PFC response to TNP-KLH than does simultaneous administration of the ascites fluid and antigen. Most importantly, the effect is not obtained in athymic mice. Therefore, it seems likely that the enhancing effect of IgD on the immune response is T cell mediated. This interpretation is also in agreement with the present observations that suggest a greater enhancement of TD than of TI responses in IgD-producing, plasmacytoma-bearing mice. Although primary responses to TI antigens are also enhanced by TEPC-1017, this effect is not seen with TEPC-1033. Both tumors enhance the secondary response to the TI antigen TNP-BA, but this may reflect an effect on the priming for the secondary response, which is known to be a T-dependent aspect of the immune response even to TI antigens (25).

There is abundant evidence (11–14) for the existence of isotype-specific T

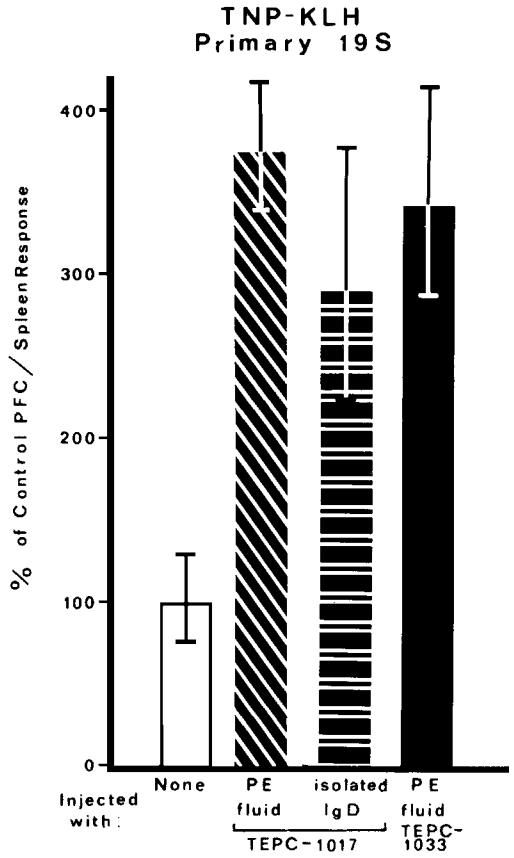

FIGURE 4. Effect of pretreatment with IgD-containing ascites fluid or with IgD isolated from such ascites on the primary 19S anti-TNP-KLH PFC response of BALB/c mice. The mice were injected with 1 ml IgD-containing peritoneal exudate (PE) fluid or saline ("None") on days -8, -7, -6, and -5 and received antigen on day 0. PFC per spleen were determined on day +5. The 7S response in this experiment was low and did not differ significantly between groups. n = 5, 4, 5, and 5; 100% value was 4,400 PFC/spleen.

cells, including T cells with Fc receptors for ϵ, α, γ, and μ both in humans and in mice. The evidence that such cells can function as suppressor cells in the synthesis of some isotypes is quite convincing. To explain the present results we postulate the existence of regulatory T cells that have Fc receptors for the δ chain and that can therefore, in principle, interact with any IgD-bearing B cell, although actually the interaction might be limited to those B cells whose membrane immunoglobulin is cross-linked by antigen. These hypothetical δ-specific T cells might be suppressors or helpers and our results on the effect of high levels of serum IgD can be explained either by "blindfolding" of suppressors or by stimulation of helpers. Since, however, pretreatment with IgD-containing ascites had a greater effect than did injection of ascites simultaneously with antigen, it seems more likely that the main event is the activation of a δ-specific helper T cell.

The actual mechanism of action of such a δ-specific T helper cell might be through a decrease in the biosynthesis of IgD itself. In fact, a decrease in surface

IgD, but not of sIgM, has been reported to accompany the blast cell transformation of antigen-stimulated B cells (26), and a recent publication (27) underlines the fact that a drastic reduction in IgD, but not in IgM synthesis, occurs early after the triggering of B cells with mitogen. In a previous study, we have shown a markedly enhanced antibody production in mice treated with anti-IgD from birth, in spite of reduced B cell numbers (8).

We are thus postulating the existence of a δ-specific helper T cell that acts by suppressing IgD synthesis in those B cells which bear aggregated IgD on their surface because they are in the process of reacting with antigen. We propose that this suppression of IgD synthesis results in the stimulation of B cell differentiation. The hypothesis implies that any B cell reacting with a TD antigen has, in addition to the conventional antigen-specific helper T cells, another potential source of help in the form of δ-specific T cells. Our results, showing an enhancement of antibody production in mice bearing IgD-secreting tumors or receiving IgD passively, would then be due to the stimulation of such a δ-specific T cell system.

The similarity between the effects of *B. pertussis* and of TEPC-1017 ascites fluid on the primary response, and the relative lack of a further enhancement by TEPC-1017 of the primary and secondary responses to DNP-OVA and *B. pertussis*, suggests that IgD and *B. pertussis* may enhance PFC responses through similar mechanisms. *B. pertussis* has been shown to prevent suppressor T cell activation (28), but may also have additional effects. On the other hand, endotoxin increases immune responses primarily by its effect on B cells. It is, therefore, of interest that the effects of endotoxin and TEPC-1017 were much more clearly additive than those of *B. pertussis* and TEPC-1017. Further study is needed to elucidate this most interesting, and, for plasmacytomas, paradoxical effect of IgD-secreting plasmacytomas on the humoral immune response.

Summary

Immune responses to trinitrophenylated hemocyanin (TNP-KLH), Ficoll (TNP-Ficoll), and *Brucella abortus* (TNP-BA) were examined in BALB/c mice bearing subcutaneous transplants of TEPC-1017 and TEPC-1033, the two known IgD-secreting BALB/c plasmacytomas. Both primary and secondary 19S and 7S splenic plaque-forming cell (PFC) responses in spleen to intravenously injected TNP-KLH were enhanced three to fivefold. Primary responses to TNP-Ficoll were 1.5–2 times higher than in control mice (particularly the 7S PFC response). Primary responses to TNP-BA were enhanced by TECP-1017 but suppressed by TEPC-1033, while secondary responses to TNP-BA were enhanced three to sevenfold by both tumors. Intraperitoneal injections of ascites fluid from mice bearing TEPC-1017 or TEPC-1033, or of IgD isolated from such ascites fluid, caused a similar enhancement of the primary response to TNP-KLH, as did the tumor itself, particularly when injected ~1 wk before antigen injection. IgD-containing ascites fluid had no effect on the response of athymic (nu/nu) BALB/c mice to TNP-KLH. These findings suggest the existence of an IgD-responsive immunoregulatory T cell.

The IgD-producing plasmacytomas, TEPC-1017 and TEPC-1033, were provided by Dr.

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