INDUCTION OF B CELL TOLERANCE IN VITRO TO 2,4-DINITRO-PHENYL COUPLED TO A COPOLYMER OF D-GLUTAMIC ACID AND D-LYSINE (DNP-D-GL)*

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Katz and colleagues (1, 2) have reported that the 2,4-dinitrophenyl (DNP) hapten when coupled to a copolymer of D-glutamic acid and D-lysine (D-GL) is a powerful inducer of hapten-specific immunologic unresponsiveness. DNP-D-GL can cause this effect in guinea pigs and mice, and in both unprimed and primed animals. Adoptive transfer studies have shown that the tolerance is induced within hours, is long-lasting, is not reversed by trypsinization of the lymphocytes before transfer, and is probably acting directly on DNP-specific bone marrow-derived (B) lymphocytes. In view of the emerging role of thymus-derived (T) lymphocytes in mediating B cell unresponsiveness (3–5), it seemed desirable to show unequivocally the absence of a T cell influence in the DNP-D-GL model. The present paper shows that DNP-D-GL can cause unresponsiveness in an entirely in vitro setting: (*a*) to a challenge antigen known to be T cell independent and (*b*) with cells derived from congenitally athymic ("nude") mice.

Materials and Methods

Mice.—CBA/H/Wehi male mice aged 10–12 wk and noninbred congenitally athymic ("nude") mice aged 6–8 wk were used.

Antigens.—A single batch of DNP-D-GL was used. It had an average mol wt of 50,000, a ratio of D-G:D-L of 60:40, and an average DNP substitution rate of 37 mol DNP per mol carrier. All details of preparation were as previously described (1, 2). Other DNP-protein conjugates and flagellar antigens including DNP coupled to *Salmonella adelaide* flagella, 1.5 mol DNP per monomeric unit of flagellin ($DNP_{1.5}FLA$), were prepared by standard methods as described by Feldmann (6–8).

Tissue Culture Methods.—The system used was slightly modified (6–8) from that of Marbrook (9). An inner well of 1 or 4 ml capacity communicated with a surrounding pool of 50 ml of nutrient medium via an interposed dialysis membrane. One of two methods was used to expose spleen lymphocytes to DNP-D-GL. In the first, the tolerogen at various concentrations

was mixed with 70×10^6 cells in 4 ml of medium and held at 0°C for 30 min to bind to lymphocyte receptors. The cells were then washed three times through fetal calf serum gradients and either (a) cultured in the larger 4 ml well for 24 h at 37°C without further antigen addition, harvested, rewashed three times, split into four equal 1-ml aliquots, and recultured for 3 days in 1-ml wells in the presence of 0.1 μg of DNP-FLA, or (b) immediately split into four aliquots and cultured for 3 days with 0.1 μg of DNP-FLA. In the second case, the tolerogen was present in culture with 70×10^6 viable spleen cells in 4-ml wells continuously for 6 h at 37°C. Then the cultures were washed three times, split into four 1-ml aliquots, and cultured for 3 days with DNP-FLA. Frequently, 10 μg of polymerized flagellin (POL) was included during the challenge phase as it was found to increase the in vitro response (10).

Assays for Antibody-Forming Cells.—The method of Cunningham and Szenberg (11) was used to determine numbers of plaque-forming cells (PFC). Modifications as previously described from our laboratories (6-8, 12) were introduced for measuring hapten- or protein-specific PFC. Each result cited is the mean of 4-16 cultures.

RESULTS

First, DNP-D-GL was bound on to lymphocytes at 0°C and the cells were washed and challenged with DNP-FLA immediately. The results are given in Table I. Control CBA spleen cells gave a good primary anti-DNP response in vitro, as did spleen cells from nude mice in the presence of POL, but the response of the latter in the absence of POL was smaller. We have found that POL has interesting properties in tissue cultures, allowing triggering of B cells by deaggregated fowl gamma globulin (10) and raising the numbers of "background" plaques of various specificities (J. W. Schrader, manuscript in preparation). In the present context, its main usefulness was to yield higher plaque numbers in control cultures of spleen cells from nude mice, and it is of interest that the nonresponsiveness caused by DNP-D-GL could be maintained despite the addition of this adjuvant-like material. Table I also shows that concentrations of DNP-D-GL of 10 ng/ml and above gave significant impairment of DNP-

TABLE I

Induction of Unresponsiveness to DNP-FLA by Precoating Lymphocyte Receptors with DNP-D-GL

Concentration of DNP-D-GL/ml during precoating step*	Nos. of DNP-specific PFC culture ± SE		
	CBA mice 0.1 μg DNP-FLA	"Nude" mice	
		0.1 μg DNP-FLA	0.1 DNP-FLA + 10 μg POL
0	739 ± 94	154 ± 27	406 ± 62
1 μg	0	17 ± 9	85 ± 15
100 ng	89 ± 26	69 ± 21	81 ± 14
10 ng	360 ± 50	83 ± 9	270 ± 24
1 ng	469 ± 59	88 ± 19	422 ± 52
100 pg	765 ± 63	122 ± 29	459 ± 44

* DNP-D-GL was added to spleen cells at the indicated concentration. After 30 min at 0°C the cells were washed and immediately cultured for 3 days with the antigens indicated.

specific PFC responses. DNP-D-GL could have been competitively inhibiting the binding of the immunogen DNP-FLA (8). However, this would require that DNP-D-GL remain at the lymphocyte surface and not be removed by "capping" and receptor pinocytosis (13, 14), since DNP-FLA was present continuously during the 3 day challenge period.

Accordingly, spleen cells, after initial binding of DNP-D-GL as above, were allowed 24 h at 37°C to "purge" themselves of any attached DNP-D-GL, or to undergo whatever metabolic or other event underlies the state of nonresponsiveness. They were then washed and challenged with DNP-FLA. The results are given in Table II. The tolerogen at optimal concentration is slightly less efficacious than with the former protocol.

A more standard design for in vitro tolerogenesis (8) is to incubate cells with tolerogen continuously present for 6 h at 37°C and then to wash and challenge. Accordingly, we present in Table III results of experiments using this design. The results are similar to those of Table II. In addition, inclusion

TABLE II

Unresponsiveness to DNP-FLA of Spleen Cells Maintained in Culture for 24 h after Binding DNP-D-GL

Concentration of DNP-D-GL/ml during precoating step*	Nos. of DNP-specific PFC/culture ± SE	
	CBA mice	"Nude" mice
0	655 ± 122	422 ± 98
1 μg	181 ± 50	117 ± 48
100 ng	304 ± 76	162 ± 19
10 ng	371 ± 81	152 ± 45
1 ng	446 ± 82	265 ± 43

* DNP-D-GL was present for 30 min at 0°C; the cells were then washed and cultured at 37°C for 24 h; they were then cultured for 3 days with 0.1 μg of DNP-FLA and 10 μg of POL.

TABLE III

Unresponsiveness to DNP-FLA Induced by Culturing Spleen Cells with DNP-D-GL for 6 h

Concentration of DNP-D-GL/ml during preculture*	Nos. of DNP-specific PFC/culture ± SE	
	CBA mice	"Nude" mice
0	706 ± 120	652 ± 31
10 μg	105 ± 60	47 ± 36
1 μg	209 ± 45	137 ± 58
100 ng	440 ± 78	180 ± 49
10 ng	471 ± 63	475 ± 64

* Cells were cultured for 6 h with DNP-D-GL and, after washing, were recultured for 3 days with 0.1 μg of DNP-FLA and 10 μg of POL.

of the 10 µg/ml concentration of DNP-D-GL in these experiments showed a more profound depression with this dose than with 1 µg/ml.

Specificity and Toxicity Controls.—Various experiments were performed to show that DNP-D-GL was not capable of inhibiting irrelevant immune responses. Thus, DNP-D-GL-pretreated cultures were shown to give normal PFC response against donkey red cells, sheep red cells, unconjugated POL, and fowl gamma globulin plus 10 µg of POL. Moreover, we included groups in many experiments where the pretreatment consisted of 10 µg/ml of DNP_{11} bovine serum albumin, and we repeated the observation of Feldmann (8) that such conjugates cannot cause tolerance in this in vitro system.

DISCUSSION

The chief value of these studies has been to show that DNP-D-GL can cause unresponsiveness in cell populations free of detectable T cells, such as spleen cells from nude mice. Furthermore, the clear-cut depression of the response of CBA spleen cells to an antigen, DNP-FLA, known to be T cell independent as far as the in vitro primary response is concerned, substantiates a direct effect on B cells. The studies have not, however, revealed the mechanism of unresponsiveness. Trypsinization of cells rendered unresponsive by DNP-D-GL cannot reverse the inhibition, under circumstances where simple receptor blockade can be reversed (2), but in such experiments it is not clear to what extent the lack of trypsin-digestibility of attached antigen may influence receptor digestion and antigen detachment. It will be for future experiments to determine whether DNP-D-GL renders B cells unresponsive through some irreversible metabolic pathway, or whether it simply remains so firmly attached to the membrane as to permanently prevent triggering by immunogenic antigen. On balance, the former alternative appears more likely.

The other major feature of interest in this model is that it represents the first occasion that in vitro unresponsiveness in B cells has been induced by a soluble antigen that is not a high molecular weight polymer. Previous models (polymerized flagellin, endotoxin) have involved much larger molecules with regularly repeating antigenic determinants. DNP-D-GL has a mean mol wt of 50,000. Of course, each molecule carries many DNP residues, so one could envisage efficient receptor cross-linking with DNP-D-GL. Yet highly DNP-substituted albumins and globulins are not good tolerogens in vitro (2, 8). A careful study of tolerogenicity with hapten substitution rate as the key variable would be revealing.

The present results support the view (2) that DNP-D-GL will be a useful tool in the elucidation of molecular mechanisms in at least one form of immunologic tolerance.

SUMMARY

Spleen cells from CBA or congenitally athymic ("nude") mice were pretreated with various concentrations of DNP coupled to a copolymer of D-glutamic acid

and D-lysine (DNP$_{37}$-D-GL), under various conditions of time and temperature. After washing, they were then cultured for 3 days with the direct B cell immunogen, DNP coupled to *Salmonella adelaide* flagella (DNP-FLA). Under all circumstances tried, exposure of cells to 1 μg/ml DNP-D-GL caused a 70–100% depression in the subsequent DNP-specific PFC response, and 100 ng/ml caused a lesser but still substantial effect. At the concentrations used, DNP-D-GL did not affect irrelevant antibody responses. Though cells from nude mice responded somewhat less well to DNP-FLA than those from CBA mice, no significant difference in the reaction of the two populations to the tolerogen was noted. This demonstrates that DNP-D-GL can, as previously suspected, directly cause unresponsiveness in B lymphocytes.

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