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# The effects of synthetic polymeric agents on immune responses of nude mice

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**Summary.** The influence of synthetic polymeric agents on the immune responsiveness of congenitally athymic (nude) mice was investigated by determining the effects of *in vivo* treatment with polynucleotides and polymeric haptenated antigens on splenic theta-bearing cells, on mitogen stimulation and on plaque-forming cell responses to thymic dependent and thymic independent antigens. Contrary to *in vitro* data, no evidence was obtained to demonstrate *in vivo* restoration of these immune parameters by the use of non-specific immune enhancers. Further, despite the continued release of lipopolysaccharide from the bowel, older nude mice (10 months old) demonstrated no acquisition of improved T-cell function. Nude mice responded well to the thymic independent antigen p-azobenzenearsonate-N-acetyl-tyrosylglycylglycine (A-TGG) Ficoll. Finally, the class specific responses to the thymic independent antigen DNP-Ficoll were significantly different from those of nu/+ littermate controls, indicating the importance of thymic influences upon the class switching of immunoglobulin responses.

## INTRODUCTION

Congenitally athymic mice homozygous for the recessive gene 'nu' (nude) are born without a thymus and accordingly without T-cell function (Flanagan, 1966; Pantelouris, 1973). Athymic mice are thus severely depleted in thymic-derived lymphocytes. There is strong evidence, nevertheless, indicating that they are well-endowed with T-cell precursors (Loor & Roelants, 1973; Roelants, Loor & Von Boehmer, 1975). Indeed, a small percentage (around 4–7 per cent) of theta-bearing cells can occasionally be demonstrated in the spleens of nude mice (Raff & Wortis, 1970). There is also evidence suggesting that the T-cell precursors of nude mice are subject to the action of thymic extracts and that they undergo maturation processes *in vitro* which lead to the acquisition of surface antigens usually encountered on mature T cells (Scheid, Hoffman, Komuro *et al.*, 1975; Komuro & Boyse, 1973a). Further, in many instances, polymeric substances such as polyadenylic polyuridylic acid (Poly A. Poly U), single-stranded DNA (ss DNA) and lipopolysaccharide (LPS) can effectively substitute for thymic extracts in this regard (Komuro & Boyse, 1973b; Goldstein, Scheid, Hammerling *et al.*, 1975). Similar data have been advanced which suggest that *in vitro* treatment of T-cell precursors from nude mice with thymic factors can confer not only new antigenic surface markers, but also a newly acquired capacity to cooperate with companion B cells in immune responsiveness to thymic dependent antigens (Ikehara,

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Hamashima & Masuda, 1975; Thurman, Ahmed, Strong Gershwin, Steinberg & Goldstein, 1975).

The extent to which these same types of maturational events may occur *in vivo* with polymeric agents remains uncertain. Indeed, since the usual thymic independent antigens are usually also polymeric, the concept of thymic-independent antigens has been called into question (Scheid *et al.*, 1975; Komuro & Boyse, 1973a). Thus it has been proposed that polymeric antigens, because of their unique character, function first to generate their own functional T-cell population from non-functional precursors, and that such newly arisen T cells may then cooperate with B cells to elicit antibody formation. Interestingly, polymeric agents were found to substitute effectively for thymosin, in a modified mouse rosette assay (Gershwin, Steinberg, Woody & Ahmed, 1975). Similarly, since endotoxin (LPS) is continually released in the bowel, one would expect to find some evidence of improved T-cell function in long-lived nude mice. In support of this latter hypothesis, older nude mice, in contrast to younger, are more resistant to oncogenesis by polyoma virus (Stutman, 1975).

## MATERIALS AND METHODS

### *Animals*

Congenitally athymic (nude) mice were outbred on an N NIH(S) mouse background and were obtained from the Small Animal Section, Rodent and Rabbit Production Unit, National Institutes of Health and the Experimental Animal Resource Facility, University of California School of Medicine at Davis. The natural history and immunopathology of mice from this mouse colony have previously been described (Gershwin, Merchant, Gelfand *et al.*, 1975). Nude mice were produced by the mating of nu/+ females with nu/nu males; they were housed and maintained under specific pathogen-free conditions until 4 weeks of age. Thereafter the mice were transferred to standard laboratory cages, freshly cleaned and autoclaved, and covered with a filtered lid (Whatman Qualitative filter paper, London). All mice were fed standard Purina lab chow and water brought to pH 2·8 with HCl.

### *Effect of age on the immune responsiveness of nude mice*

Groups of four to six healthy nude mice were immunized i.p. with $2 \times 10^8$ sheep red blood cells (SRBC) at 2 months, 6 months, or 10 months of age. The response to SRBC was determined by splenic plaque-forming cells (PFC) 5 days after immunization (Jacobs, Steinberg, Gordon *et al.*, 1972). Additional groups of three to five mice, at the same ages, were killed and single cell suspensions aseptically prepared from their spleens in RPMI 1640 media (Grand Island Biologic Company, Grand Island, New York). The percentage of splenic thy 1·2-bearing cells was determined by a trypan blue cytotoxicity assay (Gershwin, Chused & Steinberg, 1974); anti-thy 1·2 sera were prepared by a modification of the Reif and Allan method as previously described (Parker, Chused & Steinberg, 1974). An aliquot of the remaining cells was cultured in microtitre plates for mitogen studies (Strong, Ahmed & Thurman, 1973). To triplicate cultures from each animal were added 100 μl of each mitogen: phytohaemagglutinin-P (PHA-P) 0·1 per cent final concentration (Difco Labs, Detroit, Michigan); Con A 0·25 μg/culture (Calbiochem, San Diego, California); *E. coli* lipopolysaccharide (LPS) 50 μg/culture (Sigma Chemical Company, St. Louis, Missouri); or RPMI 1640 medium alone. Cultures were incubated for 72 h at 37° in a 5 per cent $CO_2$, 80 per cent oxygen, 15 per cent nitrogen, humidified atmosphere. Eighteen hours before harvest, each culture received 1 μCi of $^3$H-labelled methylthymidine ([$^3$H]TdR) (1·9 Ci/mmole, Schwarz-Mann, Orangeburg, New York) in 10 μl of medium. Cultures were harvested with an automated multiple sample harvester (Strong *et al.*, 1973); nu/+ mice, of identical ages, were included as positive controls.

### *Immunization of nude mice with poly I. poly C and poly A. poly U*

Groups of 8-week old nu/nu mice were immunized with polyinosinic polycytodylic acid (Poly I. Poly C) or polyadenylic polyuridylic acid (Poly A. Poly U) (Miles Laboratories, Kankakee, Illinois). Each group consisting of four to six mice received either (a) 1·0, 10 or 100 μg of polymer in borate buffer on day 0, 3, 6, 9, 12, 15, 18 and 21 or (b) 1·0, 10 or 100 μg of polymer coupled to methylated bovine serum albumin (mBSA) in borate buffer electrostatically on day 0, 3, or 6 or (c) 10, 40 or 400 μg of polymer in borate buffer emulsified with complete Freund's adjuvant on day 0, or (d) 10, 40 or 400 μg of polymer in borate buffer emulsified with complete Freund's adjuvant on day 0, or (e) 10, 40 or 400 μg of polymer coupled to

mBSA and emulsified in FCA on day 0 (Strong *et al.*, 1973). The activity of the Poly I. Poly C was confirmed by its ability to stimulate interferon production (performed by Dr Sam Baron). The activity of ssDNA and Poly A. Poly U was confirmed by the modified mouse rosette assay (Gershwin *et al.*, 1975). All injections were i.p. Nu/+ and BALB/c mice were used as controls. Mice were bled from the orbital sinus serially after immunization and the sera assayed for antigen binding capacity as previously described (Steinberg, Pincus & Talal, 1971b).

Additional groups of four to six mice were immunized i.p. with either (a) 100 $\mu$g Poly I. Poly C. in borate buffer; (b) 100 $\mu$g Poly A. Poly U in borate buffer; or (c) borate buffer only. Three days later the mice were killed and the percentages of splenic thy 1·2-bearing cells and of mitogen responsiveness were determined as above. Other groups of four to six mice were immunized i.p. with either (a) 10·0, 40·0 or 400 $\mu$g Poly I. Poly C in FCA; (b) 10·0, 40·0, or 400 $\mu$g PolyA-Poly U in FCA; (c) 10·0, 40·0 or 400 $\mu$g single-stranded DNA methylated bovine serum albumin (DNA.mBSA) in CFA; or (d) borate buffer in FCA. Half the groups were killed at 7 days, the remainder at 14 days. The percentage of splenic thy 1·2-bearing cells and of mitogen responsiveness were then determined.

*Attempts to restore immune responsiveness of nude mice with nonspecific immune enhancers*

Groups of nude mice were given i.p. injections of polynucleotides in an attempt to restore splenic PFC responsiveness to SRBC. Groups of four to six mice were treated with either (a) 100 $\mu$g Poly I. Poly C; (b) 100 $\mu$g Poly A. Poly U; (c) 100 $\mu$g ssDNA.mBSA; (d) 10, 40 or 400 $\mu$g Poly I. Poly C in FCA; (e) 10·0, 40 or 400 $\mu$g Poly A. Poly U in FCA; or (f) 10·0, 40 or 400 $\mu$g ssDNA.mBSA in FCA. Ten days thereafter, the mice were immunized i.p. with $2 \times 10^8$ SRBC. The splenic PFC responses and the percentages of thy 1·2-bearing cells were determined 4 days later.

In addition, groups of five or six nu/nu and littermate nu/+ mice were injected i.p. with 500 $\mu$gm Poly A. Poly U 4 h before, or 30–50 $\mu$g LPS (salmonella enteritidis W-3126 endotoxin) Difco Laboratories, Detroit, Michigan) 2 or 8 h before i.p. immunization with $10^8$ burro red blood cells. A polymeric haptenated antigen, p-azobenzene-sulfonate-N-acetyl-tyrosylglycylglycine (S-TGG) on aminoethylcarbamylmethyl (AECM) Ficoll ($S_{61}$ AECM-Ficoll) was also used in these experiments either alone or in conjunction with the immune enhancers. All mice were killed on the 4th day and their IgM secreting splenic PFC levels determined (Merchant & Petersen, 1968).

A similar experiment was performed using dinitrophenyl-$\beta$-alanylglycylglycine-conjugated keyhole limpet haemocyanin ($DAGG_{22}$ KLH) as the thymic-dependent antigen, and p-azobenzenearsonate-N-acetyltyrosylglycylglycine (A-TGG) Ficoll ($A_{45}$ AECM Ficoll) as the thymic-independent antigen. The haptenated Ficoll antigens were kindly provided by Dr John Inman (Inman, 1975).

*DAGG-reactive plaque-forming cells*

Nu/nu and nu/+ mice 6 weeks of age were immunized with 50 $\mu$g of the thymic-independent carrier Ficoll conjugated directly with the dinitrophenyl group only ($DNP_{25}$-Ficoll) in saline i.p. on day 0. Control mice received saline only. One-fourth of each group was boosted on day 10 with 50 $\mu$g $DNP_{25}$-Ficoll i.p. Groups of six mice were assayed on days 4, 7, 10 and 13 for splenic plaque-forming cells (PFC) reactive with DAGG as previously described (Inman, Merchant, Clafin *et al.*, 1973). IgM-secreting cells were enumerated by a modification of the direct Jerne plaque method (Merchant & Petersen, 1968). Goat anti-mouse IgM antibody diluted 1:400 was used in separate assays to suppress these direct plaques so that indirect plaques of the $IgG_1$, $IgG_2$, and IgA classes could be enumerated by enhancement with class-specific rabbit antisera. These class-specific antisera were a gift from Dr Richard Asofsky.

## RESULTS

### Effect of age on immune responsiveness of nude mice

There were no significant differences in nude mice at 2, 6 or 10 months of age with respect to percentages of splenic thy 1·2-bearing cells, response to SRBC immunization or stimulation by the mitogens Con A or PHA-P (Tables 1 and 2).

Nude mice at all ages responded to the B-cell mitogen, lipopolysaccharide, as well as nu/+ littermates. In contrast, only nu/+ mice responded well to both SRBC and the T-cell mitogens Con A and PHA-P.

**Table 1.** Effect of age on immune responsiveness

| Mice* | Age (months) | Per cent thy 1,2-bearing cells | Plaques/$10^6$ cells† |
|---|---|---|---|
| nu/nu | 2 | 5±2 | 20±4 |
| nu/+ | 2 | 31±4 | 475±95 |
| nu/nu | 6 | 6±3 | 15±7 |
| nu/+ | 6 | 33±5 | 680±120 |
| nu/nu | 10 | 4±2 | 22±9 |
| nu/+ | 10 | 30±6 | 500±40 |

* Four to six mice per group.
† All mice immunized with $2 \times 10^8$ SRBC i.p. and assayed on day 4± s.e.m.

## Mitogen responsiveness of nude mice after polynucleotide immunization

The optimal dose for Poly I. Poly C and for Poly A. Poly U responsiveness was 10 μg in buffer or 40 μg in adjuvant. The response data for immunization at these dosages is provided in Table 3. A dose of 100 μg in buffer was essentially identical to 10 μg. The thymic independent nature of the antigens Poly I. Poly C and Poly A. Poly U can be seen in Table 3. A total of 80 μg of Poly I. Poly C, given as 10 μg injections 3 days apart, led to significant antibody production (Table 3). Even greater quantities of antibody were produced in response to Poly A. Poly U. Electrostatic complexes of Poly I. Poly C and mBSA led to low levels of antibody. This was not much improved upon by incorporation into adjuvants. Throughout Poly A. Poly U was more immunogenic than Poly I. Poly C. These results were almost identical to those for nu/+ and BALB/c mice (data not shown).

Treatment with polynucleotides increased the background (autostimulation) of splenic cultures from both nu/nu and nu/+ mice, but it did not increase the relative numbers of thy 1·2-bearing cells, nor did it increase the stimulatory effects of either Con A or PHA (Table 4). The percentage of thy 1·2-bearing cells did not change appreciably following polynucleotide stimulation Table 5). The response to SRBC was unaltered following treatment with Poly I.C or Poly A.U. However, it was significantly increased with either ssDNA.mBSA or ssDNA. mBSA.FCA ($P < 0{\cdot}05$ Student's *t*-test). It is important to note that this increase was unaccompanied by a rise in thy 1·2 cells.

## PFC responsiveness of nude mice after stimulation with non-specific immune enhancers

Poly A. Poly U, LPS, $S_{61}$ AECM Ficoll or combinations of these polymeric agents were given to groups of nude and nu/+ mice at 2–8 h intervals prior to immunizations with BRBC, a thymic-dependent antigen in an effort to enhance nude responsiveness to BRBC. The highest BRBC reactive PFC response in nu/nu mice was fifteen PFC/$10^6$ spleen cells in a group of nude mice given BRBC without treatment with 'enhancers' (Table 6). Nu/nu mice mounted less vigorous PFC responses to $S_{61}$ AECM Ficoll than nu/+ littermates (Table 6). However, the nu/nu mice did mount obvious S-TGG specific PFC responses in all experiments. Most importantly, S-TGG specific PFC responses of nu/nu mice were not enhanced by simultaneous *in vivo* treatment with large quantities of Poly A. Poly U or LPS (Table 6).

($DAGG_{22}$) KLH, a hapten-derived protein bearing enlarged haptenic side chains, was used as another indicator of responsiveness to thymic

**Table 2.** Effect of age on mitogen responsiveness

| Mice* | Age (months) | Control | Con A | PHA-P† | LPS† |
|---|---|---|---|---|---|
| nu/nu | 2 | 310±45 | 405±60 | 380±70 | 11,500±1300 |
| nu/+ | 2 | 430±70 | 62,500±8000 | 98,000±11,000 | 8300±900 |
| nu/nu | 6 | 430±60 | 370±40 | 450±75 | 9850±1200 |
| nu/+ | 6 | 290±90 | 73,400±17,500 | 89,200±21,200 | 10,500±1400 |
| nu/nu | 10 | 380±50 | 490±150 | 510±90 | 14,700±1900 |
| nu/+ | 10 | 410±70 | 65,700±13,400 | 90,300±19,200 | 9100±1800 |

* Four to six mice per group.
† Response to mitogen expressed as c.p.m. in stimulated cultures minus c.p.m. in unstimulated cultures± s.e.m.

**Table 3.** Antibody response of nude mice to immunization with Poly A. Poly U or Poly I. Poly C with or without carrier protein or adjuvant

| Method of immunization* | Total dose (μg) | Day† | Immunogen: Poly I. Poly C‡ | Immunogen: Poly A. Poly U‡ |
|---|---|---|---|---|
| In borate buffer | 0 | 0 | <0·2 | <0·2 |
| | 20 | 4 | 1·0 | 3·6 |
| | 30 | 7 | 2·7 | 3·9 |
| | 80 | 21 | 1·7 | n.d. |
| With MBSA in buffer | 0 | 0 | <0·2 | n.d. |
| | 80 | 4 | 1·1 | n.d. |
| | 120 | 8 | 0·9 | n.d. |
| With MBSA in FCA | 40 | 4 | 1·3 | n.d. |
| | 40 | 8 | 2·2 | |
| In FIA | 40 | 4 | 0·2 | 1·1 |
| | 40 | 14 | 2·1 | n.d. |
| | 40 | 21 | 1·6 | n.d. |
| In FCA | 40 | 4 | 0·2 | 0·3 |
| | 40 | 14 | 0·4 | 2·6 |
| | 40 | 28 | 1·0 | 3·5 |
| In FCA, boosted 1 month later in FIA | 80 | 35 | 1·1 | 5·7 |

n.d. = Not determined.

* Four to six mice per group; immunization schedules in text.

† Time of assay after initiation of immunization schedule.

‡ Antigen-binding capacity (μg/ml); data shown only for optimal dose: 10 μg in buffer and 40 μg in adjuvant.

**Table 4.** Splenic mitogen responsiveness of mice after polynucleotide immunization

| Mice | Treatment† | Total dose (μg)* | Control† (c.p.m.) | Con A† (c.p.m.) | PHA-P† (c.p.m.) | Per cent thy 1·2-bearing cell ⩾s.e.m. |
|---|---|---|---|---|---|---|
| nu/nu† | Borate buffer | 0 | 530±60 | 610±70 | 540±30 | 2±1 |
| nu/+ | Borate buffer | 0 | 420±70 | 53,500±17,400 | 83,700±21,100 | 33±4 |
| nu/nu | Poly I. Poly C | 100 | 670±110 | 600±80 | 430±100 | 3±2 |
| nu/+ | Poly I. Poly C | 100 | 980±230 | 57,600±14,300 | 95,300±18,100 | 30±5 |
| nu/nu | Poly A. Poly U | 100 | 1050±210 | 690±90 | 670±105 | 0 |
| nu/+ | Poly A. Poly U | 100 | 1260±140 | 58,100±12,300 | 82,700±13,500 | 31±3 |
| nu/nu‡ | Poly I. Poly C. FCA | 40 | 730±80 | 780±130 | 810±150 | 4±2 |
| nu/+ | Poly I. Poly C. FCA | 40 | 960±140 | 63,500±16,200 | 89,100±11,200 | 32±6 |
| nu/nu | Poly A. Poly U. FCA | 40 | 910±170 | 830±270 | 710±190 | 2±1 |
| nu/+ | Poly A. Poly U. FCA | 40 | 1430±270 | 63,100±19,200 | 79,600±14,300 | 32±3 |
| nu/nu | Borate buffer in FCA | 0 | 410±60 | 430±80 | 610±130 | 0 |
| nu/+ | Borate buffer in FCA | 0 | 515±75 | 62,500±12,400 | 76,500±19,400 | 30±5 |
| nu/nu | ssDNA in BSA, FCA | 40 | 530±110 | 470±80 | 560±90 | 2±1 |
| nu/+ | ssDNA in BSA, FCA | 40 | 400±120 | 56,600±11,500 | 71,200±24,500 | 35±3 |

* See text for details.

† Mice killed 3 days after immunization.

‡ Mice killed 14 days after immunization; results for mice killed 7 days after immunization were almost identical.

**Table 5.** Attempts to restore thy 1·2-bearing cells and the SRBC response in nu/nu spleens by treatment with polynucleotides

| Mice* | Treatment† | Total dose (μg)† | % thy 1·2 ± s.e.m. | Plaques/$10^6$ cells |
|---|---|---|---|---|
| nu/nu | — | — | 3±2 | 49 |
| nu/+ | — | — | 33±5 | 700 |
| nu/nu | Poly I. Poly C | 100 | 4±2 | 12 |
| nu/nu | Poly I. Poly C. FCA | 40 | 3±1 | 52 |
| nu/nu | Poly A. Poly U | 100 | 5±3 | 30 |
| nu/nu | Poly A. Poly U. FCA | 40 | 4±2 | 28 |
| nu/nu | Borate FCA | — | 3±2 | 67 |
| nu/nu | ssDNA mBSA | 100 | 2±1 | 117 |
| nu/nu | ssDNA.mBSA FCA | 40 | 4±2 | 99 |

* Four to six mice/group.

† See text for details. Mice were immunized with polynucleotides on day 0, with SRBC on day 10 and their spleen cells were assayed on day 14.

**Table 6.** Splenic PFC responses of nude and nu/+ mice stimulated with non-specific immune enhancers*

| Phenotype† | Treatment with polymeric agent | Hours before BRBC | $10^8$ BRBC i.p. | BRBC PFC/$10^6$ | BRBC PFC/Spleen | S-TGG PFC/$10_6$ | S-TGG PFC/Spleen |
|---|---|---|---|---|---|---|---|
| Nude | — | — | — | 4 | 640 | 1 | 160 |
| nu/+ | — | — | — | 2 | 390 | 1 | 195 |
| Nude | — | — | + | 6 | 580 | 1 | 60 |
| nu/+ | — | — | + | 102 | 10,240 | 1 | 80 |
| Nude | 30 μg LPS‡ i.p. | 8 | + | 9 | 880 | 4 | 420 |
| nu/+ | 30 μg LPS‡ i.p. | 8 | + | 79 | 7140 | 8 | 760 |
| Nude | 1 μg $S_{61}$ AECM Ficoll§ i.p. | 8 | + | 9 | 700 | 83 | 6300 |
| nu/+ | 1 μg $S_{61}$ AECM Ficoll§ i.p. | 8 | + | 227 | 22,760 | 186 | 18,600 |
| Nude | — | — | — | 5 | 1280 | 2 | 512 |
| nu/+ | — | — | — | 3 | 480 | 1 | 160 |
| Nude | — | — | + | 15 | 2080 | 2 | 240 |
| nu/+ | — | — | + | 258 | 56,500 | 0 | 0 |
| Nude | 500 μg Poly AU i.p. | 4 | + | 7 | 1040 | 0 | 0 |
| nu/+ | 500 μg Poly AU i.p. | 4 | + | 335 | 70,400 | 3 | 760 |
| Nude | 1 μg $S_{61}$ AECM Ficoll i.p. | 4 | + | 4 | 840 | 25 | 4680 |
| nu/+ | 1 μg $S_{61}$ AECM Ficoll i.p. | 4 | + | 287 | 60,640 | 91 | 19,300 |
| Nude | 500 μg Poly AU i.p. and 1 μg $S_{61}$ AECM Ficoll i.p. | 4 | + | 6 | 1200 | 19 | 3620 |
| nu/+ | 500 μg Poly AU i.p. and 1 μg $S_{61}$ AECM Ficoll i.p. | 4 | + | 374 | 70,400 | 76 | 14,420 |
| Nude | — | — | — | 3 | 420 | 1 | 140 |
| nu/+ | — | — | — | 4 | 440 | 1 | 110 |
| Nude | — | — | + | 5 | 530 | 3 | 30 |
| nu/+ | — | — | + | 368 | 53,310 | 2 | 220 |
| Nude | 50 μg LPS i.p. | 2 | + | 8 | 1070 | 1 | 140 |
| nu/+ | 50 μg LPS i.p. | 2 | + | 224 | 38,560 | 2 | 40 |
| Nude | 1 μg $S_{61}$ AECM Ficoll i.p. | 2 | + | 9 | 940 | 53 | 5560 |
| nu/+ | 1 μg $S_{61}$ AECM Ficoll i.p. | 2 | + | 274 | 25,760 | 130 | 13,780 |
| Nude | 50 μg LPS i.p. and 1 μg $S_{61}$ AECM Ficoll i.p. | 2 | + | 7 | 810 | 27 | 3120 |
| nu/+ | 50 μg LPS i.p. and 1 μg $S_{61}$ AECM Ficoll i.p. | 2 | + | 314 | 44,960 | 35 | 5000 |

* Mean fourth day primary IgM PFC responses.

† Five or six mice/group.

‡ S. enteritidis endotoxin, W-3126, Difco.

§ p-azobenzenesulphonate-*N*-acetyltyrosylglycylglycine (S-TGG) derived to aminoethycarbamylmethyl (AECM) Ficoll at sixty-one haptenic substituents per 400,000 mol. wt of AECM Ficoll.

**Table 7.** Fourth day primary splenic IgM PFC responses in nude and nu/+ mice stimulated with thymic dependent and thymic independent antigens

| Phenotype* | 10 μg A-$TGG_{45}$AECM Ficoll i.p. at −2 h | 10 μg $DAGG_{22}$ KLH i.p. at 0 h | DAGG PFC/$10^6$ | DAGG PFC/spleen | A-TGG PFC/$10^6$ | A-TGG PFC/spleen |
|---|---|---|---|---|---|---|
| Nude | − | − | 4 | 790 | 2 | 410 |
| Nu/+ | − | − | 4 | 630 | 1 | 120 |
| Nude | − | + | 32 | 7080 | 3 | 620 |
| Nu/+ | − | + | 99 | 17,450 | 2 | 340 |
| Nude | + | − | 10 | 1740 | 24 | 4330 |
| Nu/+ | + | − | 10 | 1380 | 22 | 3100 |
| Nude | + | + | 28 | 6020 | 21 | 4420 |
| Nu/+ | + | + | 116 | 15,850 | 27 | 3730 |

*Five to six mice per group.

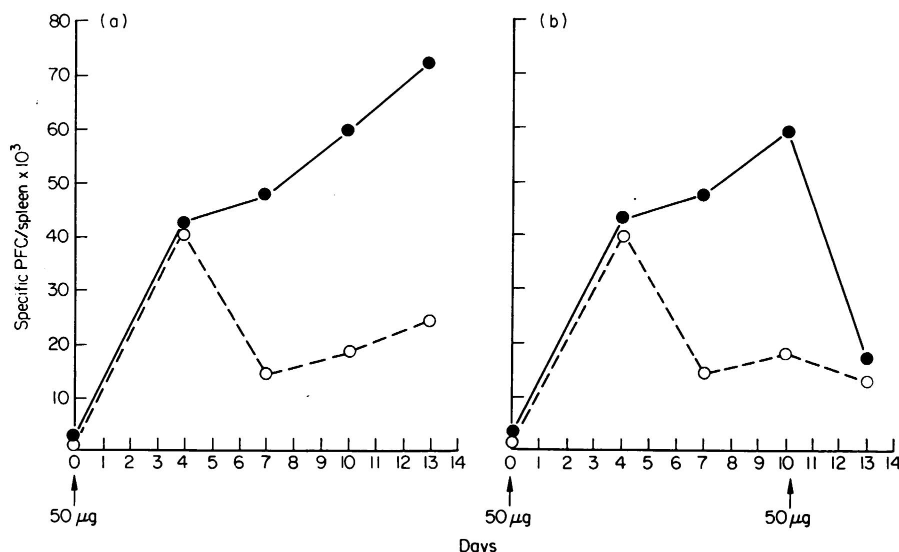


**Figure 1.** IgM PFC reactive with DAGG in DNP–Ficoll-immunized mice. Groups of nu/nu (●——●) and nu/+ (○ – – ○) mice were immunized with 50 μg of $DNP_{25}$ Ficoll i.p. (a) and boosted with 50 μg on day 10 (b). The specific IgM plaque-forming cells on pools of six mouse spleens were quantified from each group on days 0, 4, 7, 10 and 13.

dependent antigens (Table 7). It did not appear to be as thymic dependent as would be expected for DNP-KLH since nu/nu mice responded to it approximately one third as well as did nu/+ littermate controls. However, treatment of nu/nu mice with 10 μg of the polymeric antigen $A_{45}$ AECM Ficoll permitted specific A-TGG responsiveness without augmenting the coexistent PFC response to $(DAGG)_{22}$ KLH (Table 7). Thus, polymeric agents have consistently failed to augment coexistent 4th day thymic dependent IgM immune responses.

**Kinetics of DAGG-reactive PFC. Responses in nu/nu and nu/+ mice**

The kinetics of class-specific PFC responses were determined in nu/nu and in nu/+ mice in order to compare similarities or differences which might be referable to T-cell regulation. Following immunization with 50 μg $DNP_{25}$-Ficoll, nu/+ mice had a peak IgM PFC response on day 4 followed by a decline on day 7. The nu/+ IgG1, IgG2 and IgA PFC responses tended to increase gradually both prior to and following the IgM decline (Figs 1–4). In contrast,

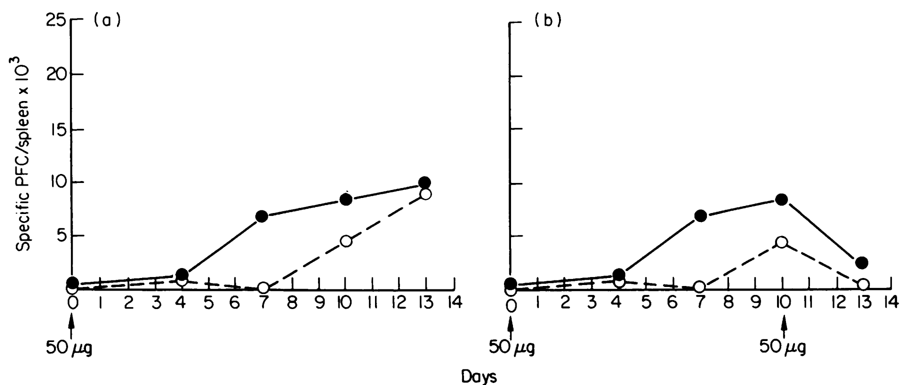


**Figure 2.** Igl PFC reactive with DAGG in DNP–Ficoll-immunized mice. Groups of nu/nu (●——●) and nu/+ (○ - - ○) mice were immunized with 50 µg of $DNP_{25}$ Ficoll i.p. (a) and boosted with 50 µg on day 10 (b). The specific IgG1 plaque-forming cells were quantified on pools of six mouse spleens from each group on days 0, 4, 7, 10 and 13.

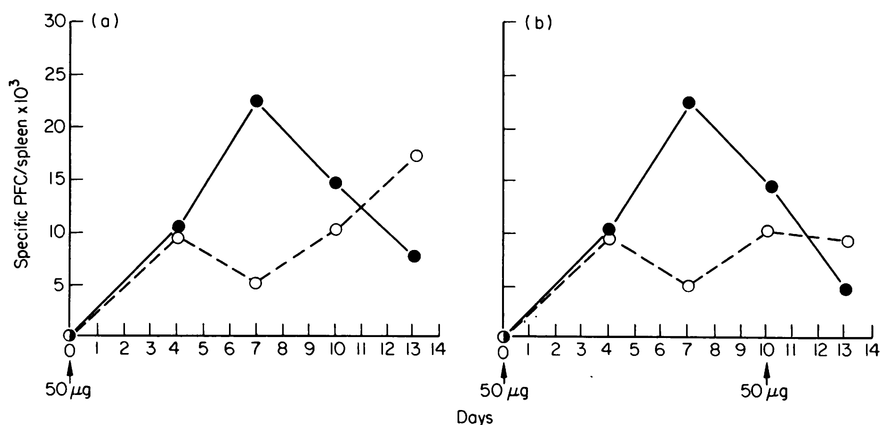


**Figure 3.** IgG2 PFC reactive with DAGG in DNP–Ficoll-immunized mice. Groups of nu/nu (●——●) and nu/+ (○ - - ○) mice were immunized with 50 µg of $DNP_{25}$ Ficoll i.p. (a) and boosted with 50 µg on day 10 (b) and the specific IgG2 plaque-forming cells were quantified on pools of six mouse spleens from each group on days 0, 4, 7, 10 and 13.

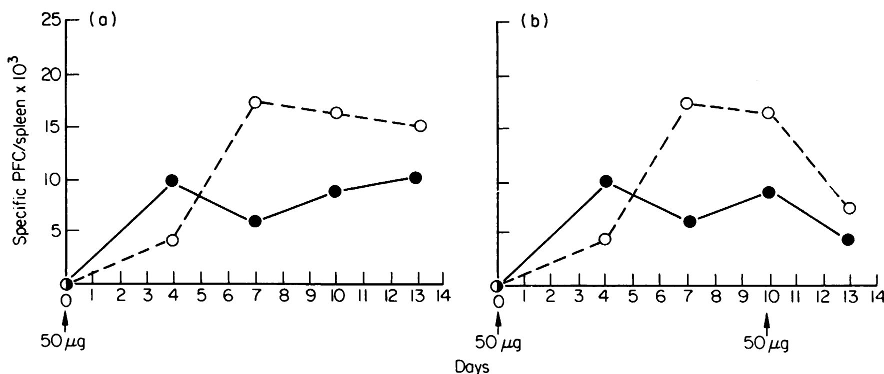


**Figure 4.** IgA PFC reactive with DAGG in DNP-Ficoll-immunized mice. Groups of nu/nu (●——●) and nu/+ (○ - - ○) mice were immunized with 50 µg of $DNP_{25}$ Ficoll i.p. (a) and boosted with 50 µg on day 10 (b). The specific IgA plaque-forming cells were quantified on pools of six mouse spleens from each group on days 0, 4, 7, 10 and 13.

IgM PFC of nude mice continued to rise after immunization and did not exhibit the decline observed with nu/+ controls (Fig. 1). In nude mice, $IgG_1$ and $IgG_2$ PFC peaked at about day 8. IgG1 held nearly constant while IgG2 began to decline (Figs 2 and 3). IgA PFC peaked on day 4 and then remained relatively constant. All mice, nu/nu and nu/+, exhibited a reduction in splenic PFC of all classes immediately following a 50 $\mu$gm $DNP_{25}$ Ficoll boost on day 10. The precise cellular significance of this post-boost reduction is uncertain; however, it was detected in assays conducted on the same day and under the same conditions as those which demonstrated continued elevation of IgM PFC responses in 13 day nu/nu mice immunized once only. If thymic independent antigens serve to generate their own regulatory T-cell populations *in vivo*, then it appears that the responses of nude mice are regulated in a manner quite different from the responses occurring in nu/+ mice which are subject to intrinsic T-cell regulation.

## DISCUSSION

The restoration of T-cell surface markers *in vitro* by thymic extracts and by synthetic polymeric agents has been demonstrated in nude mice (Scheid *et al.*, 1975; Komuro & Boyse, 1973a; Komuro & Boyse, 1973b; Goldstein *et al.*, 1975). If such events occur to any significant extent *in vivo*, polymeric agents and polymeric thymic-independent antigens might be expected to enhance the capacity of nude mice to mount a coexistent response to typical thymic dependent antigens. Thus, in the present series of experiments, pretreatment of nude mice with polymeric agents followed by BRBC immunization should increase their PFC response to this thymic dependent antigen. *In vivo* treatment with polymeric agents did not, however, enhance the capacity of nude mice to respond to BRBC.

Nude mice mounted generally less vigorous PFC responses to $S_{61}$ AECM Ficoll than nu/+ littermates; nonetheless, the responses of nude mice were many fold above that of background S-TGG specific activities. Most importantly, modest S-TGG specific PFC responses of nu/nu mice to $S_{61}$ AECM Ficoll were not enhanced by prior *in vivo* treatment with 500 $\mu$g Poly A. Poly U or by 50 $\mu$g LPS. Accordingly, provision of large excesses of polymeric agents failed to augment an otherwise autonomous thymic independent PFC response in nu/nu mice. Thus, coexistent treatment of nu/nu mice with polymeric agents failed to enhance the response to BRBC and also failed to augment an otherwise thymic-independent response to $S_{61}$ AECM Ficoll.

A similar result was obtained with $(DNP\text{-}AGG)_{22}$ KLH as the thymic dependent antigen and A-$TGG_{45}$ AECM Ficoll as the thymic independent antigen. Treatment of nu/nu mice with 10 $\mu$g of $A_{45}$ Ficoll permitted specific A-TGG responsiveness without augmenting coexistent PFC response to $(DNP\text{-}AGG)_{22}$ KLH. In these experiments, polymeric agents, including even polymeric thymic independent antigens, consistently failed to augment concomitant thymic dependent immune responses.

Polynucleotides have similarly been reported to be capable of restoring both thy 1·2 positivity and the response to SRBC (Scheid, 1975). In the test conditions employed here, pretreatment *in vivo* with Poly A. Poly U, Poly I. Poly C or single stranded DNA all consistently failed to restore thy 1·2 positivity. ssDNA, in contrast, did minimally augment the response to SRBC. This observation is consistent with previous data that demonstrate that ssDNA is a better adjuvant than Poly I. Poly C or Poly A. Poly U. It is interesting to note that the adjuvant effect is independent of alterations in quantity of thy 1·2 cells. In addition, because LPS is continually released from the gut, one would expect an improvement in T-cell function with age; yet there is no evidence from the present data that thy 1·2 positivity or increased capacity to respond to thymic dependent antigens or T-cell mitogens is so acquired. This observation is important because of *in vitro* observations that treatment of precursor cells from murine spleen or bone marrow with LPS leads to expression of both TL and thy 1·2 antigen (Scheid *et al.*, 1975). Thus further studies must be entertained to address whether initiation of T-cell differentiation *in vitro* as evidenced by appearance of cell surface markers, implies physiological T-cell activity.

The nude S-TGG specific response and the differences in DAGG reactive PFC following DNP-Ficoll immunization are consistent with data presented elsewhere (Gershwin *et al.*, 1975). Nude mice have markedly elevated background PFC to several synthetic haptens (Gershwin *et al.*, 1975). Additionally, they have abnormal immunoglobulin levels (Gershwin *et al.*, 1975; Bankhurst *et al.*, 1975). It has been suggested that the elevated levels of IgM in nude mice as well as the elevated background PFC

responses to synthetic haptens is due to the absence or malfunction of a thymus-derived suppressor-cell population (Bankhurst *et al.*, 1975). The lack of normal transition from IgM to IgG PFC in nude mice following DNP-Ficoll immunization may also be secondary to absence of a regulatory T-cell population.

Whether non-specific enhancers can gain effective access to T-cell precursors *in vivo* is uncertain. The failure of ageing nude mice to acquire increased T-cell function suggests, however, that 'spontaneous' conversion of T-cell precursors to functional T-helper cells is normally a rare event *in vivo*. Until procedures can be devised which permit non-thymic products to convert precursors into functional T cells *in vivo*, some question must remain as to whether non-specific maturation of T cells *in vitro* represents anything more than an interesting laboratory artifact. Thus, true non-specific restoration of T-cell function in nude mice must await other immunotherapeutic approaches.

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