THYMUS INDEPENDENCE OF A COLLAGEN-LIKE SYNTHETIC POLYPEPTIDE AND OF COLLAGEN, AND THE NEED FOR THYMUS AND BONE MARROW-CELL COOPERATION IN THE IMMUNE RESPONSE TO GELATIN*

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The immune response to most antigens requires cooperation between thymus-derived and bone marrow cells (1–3). It has also been suggested that a third cell type, the macrophage, is involved in the induction of humoral antibody response (4, 5). A cell to cell interaction has been established for synthetic polypeptide antigens as well (6–8).

In the last few years it has been reported that for some immunogens an efficient antibody response can be obtained in the absence of thymocytes. These so-called thymus-independent antigens include polymerized flagellin (9, 10), pneumococcal polysaccharide type S III (11), *Escherichia coli* lipopolysaccharide (12, 13), polyvinylpyrrolidone (13), and the synthetic double-stranded polyribonucleotide poly(I)·poly(C) (14). We have shown recently that those synthetic polypeptides derived from multichain polyproline (polyPro--polyLys) and containing D-amino acid residues (15), which are slowly metabolized (16), are thymus independent (17).

It appears that the common features to all the above thymus-independent antigens are repeating identical antigenic determinants, slow metabolism, or a combination of both. In this connection it was of interest to find out to what extent the steric conformation of the immunogen plays a role in the need for cellular cooperation, in order to obtain an efficient immune response. Studies on synthetic polypeptides in mice, and the cellular analysis of their antibody responses, were performed with antigens obtained by random polymerization of *N*-carboxyamino acids anhydrides (8, 18, 19). These synthetic models do not possess a unique three-dimensional conformation. The role of antigenic conformation in immunogenicity and antigenic specificity has been investigated previously by using synthetic antigens possessing an ordered periodic sequence (20–24).

The synthetic ordered polymer of the tripeptide L-prolyl-glycyl-L-proline, designated $(Pro\text{-}Gly\text{-}Pro)_n$, was shown to resemble collagen in its three-dimensional structure both in solution (25) and in the solid state (26). Immunochemical studies have shown that the characteristic triple-helical collagen-like conformation of this polymer is crucial in defining its antigenic specificity. Thus, antibodies to this collagen-like

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polymer cross-react with other synthetic collagen-like polymers, whereas only a poor cross-reaction was observed with a random copolymer, $(Pro^{66}, Gly^{34})_n$, which is similar to the ordered copolymer in its amino acid composition, but is not collagen-like (24). Moreover, antibodies to the ordered polymer $(Pro\text{-}Gly\text{-}Pro)_n$ cross-reacted with several collagens from different animal species (23, 27, 28).

In view of the above results it was of interest to establish the role of amino acid sequence and three-dimensional conformation in determining the cell types involved in the immune response to the ordered $(Pro\text{-}Gly\text{-}Pro)_n$ and to collagen on the one hand, and to compare it with the response to the random polymer $(Pro^{66}, Gly^{34})_n$ and to gelatin, on the other hand.

The experiments described in this report demonstrate that the collagen-like $(Pro\text{-}Gly\text{-}Pro)_n$ is a thymus-independent antigen, whereas for an efficient response to the random $(Pro^{66}, Gly^{34})_n$, cooperation between thymus and marrow cells is necessary. Similarly, the native triple-helical collagen was found to be an immunogen independent of the thymus, whereas in order to elicit a response to its denatured product, gelatin, thymus cells are required.

Materials and Methods

Mice.—Inbred mice, 8–12 wk of age, of the C57BL/6, C3H.SW, BALB/c, DBA/2, AKR/Cu, C3H/HeJ, DBA/1, SWR, and SJL/J strains were obtained from the Experimental Animal Unit, The Weizmann Institute of Science.

Antigens.—The ordered collagen-like polymer $(Pro\text{-}Gly\text{-}Pro)_n$ (Mol Wt 6,300) and the random copolymer $(Pro^{66}, Gly^{34})_n$ (mol wt 6,800) were prepared as previously described (23–25). The conjugates $(Pro\text{-}Gly\text{-}Pro)_n$- RNase and $(Pro^{66}, Gly^{34})_n$-RNase were prepared by conjugation of the respective polymer (average molecular weight of about 2,000) with RNase by means of water soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Ott Chemical Co., Muskegon, Mich.] (24). Conjugates of the tripeptide Pro-Gly-Pro with ovalbumin or RNase, Pro-Gly-Pro-ovalbumin and Pro-Gly-Pro-RNase, were prepared as described elsewhere (24). Acid-soluble collagen from rat tail tendon was prepared according to Piez et al. (29). Rat tail tendon gelatin was prepared by heat denaturation of rat tail tendon collagen in phosphate-buffered saline (PBS; 0.14 M NaCl-0.01 M sodium phosphate buffer, pH 7.4) for 20 min at 45°C. Reduced and carboxymethylated (RCM) *Ascaris* cuticle collagen (30) was a gift from Dr. W. F. Harrington.

Cell Transfers and Immunization.—Recipient mice of both sexes, 10-12 wk of age, were exposed to 750 rad of ^{60}Co γ-irradiation. Cell suspensions were prepared from the thymus and bones of the hind legs of 8–10-wk old donor mice as described elsewhere (6). The concentration of each cell suspension was estimated by repeated sample counting of nucleated cells using a hemocytometer. The irradiated mice were injected via the tail vein with 10^8 thymocytes, 2×10^7 marrow cells, or with a mixture of 10^8 thymocytes and 2×10^7 marrow cells, obtained from syngeneic donors. The recipients were immunized intraperitoneally with 10 μg of the immunogen in complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.), 24 h after cell transfer. 2 wk after cell transfer, the recipients were bled and sacrificed, and their spleens were removed and assayed for hemolytic plaque-forming cells.

Intact mice were immunized with 1, 10, or 100 μg of the immunogen in complete Freund's adjuvant intradermally into the hind footpads in 0.06 ml. 3 wk later the mice were boosted with the same amount of the immunogen in PBS. 10 days after the second injection the mice were bled. All sera were assayed individually by passive microhemagglutination assay.

Immunological Assays.—

Passive microhemagglutination assay: Sheep erythrocytes were formalinized, tanned (31), and coated with collagen, gelatin, $(Pro\text{-}Gly\text{-}Pro)_n$-RNase, $(Pro^{66}, Gly^{34})_n$-RNase, and Pro-Gly-Pro-RNase. The concentrations of the antigens used for the coating were 10 μg/ml for collagen and gelatin, and 2 mg/ml for the conjugates. The hemagglutinations were performed on disposable microtiter plates (Cooke Engineering Co., Alexandria, Va.) by 2-fold serial dilutions of antisera in PBS containing 1% normal rabbit serum. The plates were incubated at 20°C and read after 2 h and 18 h later.

Hemolytic plaque-forming cells assay: Direct plaque-forming cells were measured using sheep red blood cells (SRBC) coated with collagen, gelatin, $(Pro\text{-}Gly\text{-}Pro)_n$-RNase, $(Pro^{66}, Gly^{34})_n$-RNase, and Pro-Gly-Pro-RNase. Collagen and gelatin were attached to SRBC treated with tannic acid (antigen concentration, 10 μg/ml). The conjugates were attached to SRBC by incubating equal volumes of packed SRBC, chromium chloride (2 mg/ml), and the antigen (10 mg/ml) for 5 min at room temperature. The SRBC were washed, after coating, three times in PBS and diluted to a final concentration of 10% in PBS. The assay was performed as previously described in reference 32, according to Jerne et al. (33).

RESULTS

The Immune Response of Inbred Mouse Strains to Collagen, Gelatin, and Related Synthetic Polypeptides.—Inbred mouse strains were immunized with 1, 10, and 100 μg of $(Pro\text{-}Gly\text{-}Pro)_n$ (mol wt 6,300). The highest antibody titers were obtained when 10 μg of the immunogen were used. The secondary antibody titers to $(Pro\text{-}Gly\text{-}Pro)_n$ assayed with $(Pro\text{-}Gly\text{-}Pro)_n$-RNase-coated SRBC are shown in Table I. All hemagglutination titers were relatively low as

TABLE I

*Immune Response of Different Inbred Mouse Strains to Collagen, Gelatin, and Related Synthetic Polypeptides**

Mouse strain	Immunogen					
	$(Pro\text{-}Gly\text{-}Pro)_n$	$(Pro^{66}, Gly^{34})_n$	Pro-Gly-Pro-ovalbumin	RCM *Ascaris* collagen	Rat tail collagen	Rat tail gelatin
C57Bl/6	4.3 (7)‡	2.2 (5)	3.6 (6)			
C3H.SW	2.5 (10)	4.0 (6)	6.0 (6)	6.0 (6)	6.0 (6)	5.0 (5)
BALB/c	3.8 (10)	3.2 (6)	4.3 (5)	3.0 (6)	1.8 (6)	2.0 (6)
DBA/2	2.0 (10)	4.2 (6)	4.2 (6)			
AKR	2.2 (10)	2.3 (6)	5.8 (6)			
C3H/HeJ	3.9 (10)	2.5 (6)	6.1 (5)			
DBA/1	2.0 (10)		5.6 (6)			
SWR	3.7 (8)	3.0 (9)	6.0 (6)	6.0 (6)	6.0 (6)	3.6 (6)
SJL/J	2.8 (9)	2.0 (6)	5.5 (5)			

* The secondary antibody titers to $(Pro\text{-}Gly\text{-}Pro)_n$ were assayed by hemagglutination with $(Pro\text{-}Gly\text{-}Pro)_n$-RNase-coated SRBC, to $(Pro^{66}, Gly^{34})_n$ with $(Pro^{66}, Gly^{34})_n$-RNase-coated SRBC, to Pro-Gly-Pro-ovalbumin with Pro-Gly-Pro-RNase-coated SRBC, and to the collagens and gelatin with SRBC coated with the homologous immunogen, respectively.

‡ Average $\log_2$ hemagglutination titers. Numbers in parenthesis indicate the number of mice tested.

expected from the immune response of other animal species to this ordered polymer (23, 24). C57BL/6, BALB/c, C3H/HeJ, and SWR mice, which were found to be relatively the best responders, were also immunized with $(Pro\text{-}Gly\text{-}Pro)_n$ preparations of lower molecular weights (5,200 and 2,100). A similar, but slightly lower immune response was observed with these preparations.

The hemagglutination titers after immunization and boosting with $(Pro^{66}, Gly^{34})_n$, Pro-Gly-Pro-ovalbumin, RCM *Ascaris* collagen, rat tail collagen, and rat tail gelatin are also shown in Table I. It is noteworthy that some of the mouse strains did not exhibit the same magnitude of response to the ordered collagen-like $(Pro\text{-}Gly\text{-}Pro)_n$ and to the random $(Pro^{66}, Gly^{34})_n$. For instance, C57BL/6 and C3H/HeJ mice, which were high responders to $(Pro\text{-}Gly\text{-}Pro)_n$, gave low antibody titers to $(Pro^{66}, Gly^{34})_n$. On the other hand, C3H.SW and DBA/2 mice were the highest responders to the random copolymer and relatively low responders to the ordered one.

Antibodies to the tripeptide Pro-Gly-Pro were obtained after immunization with a conjugate of the tripeptide, as a hapten, to a protein carrier. The titers of these antitripeptide antibodies were relatively high in most mouse strains tested (Table I).

Three mouse strains which were found to be good responders either to the ordered $(Pro\text{-}Gly\text{-}Pro)_n$ or to the random copolymer $(Pro^{66}, Gly^{34})_n$, were immunized with RCM *Ascaris* collagen, rat tail collagen, and rat tail gelatin. C3H.SW and SWR mice were high responders to the three immunogens, whereas BALB/c mice exhibited low titers (Table I).

The Role of the Thymus in the Immune Response to $(Pro\text{-}Gly\text{-}Pro)_n$, $(Pro^{66}, Gly^{34})_n$, *and Pro-Gly-Pro-ovalbumin.*—In order to establish whether thymus-bone marrow cell cooperation is needed for an efficient immune response to the ordered polypeptide $(Pro\text{-}Gly\text{-}Pro)_n$, transfer experiments were performed, using inbred mouse strains which were shown to be high responders. Irradiated recipients were injected with 10^8 thymocytes, 2×10^7 bone marrow cells, or a mixture of these two cell populations, followed by immunization with the polymer. For comparison, similar transfer experiments were performed using the random copolymer $(Pro^{66}, Gly^{34})_n$ and Pro-Gly-Pro-ovalbumin as immunogens. The results of the response frequencies obtained by the hemolytic plaque-forming cells assay and by passive microhemagglutination are shown in Table II. The immune response to $(Pro\text{-}Gly\text{-}Pro)_n$ was found to be thymus independent in the three strains tested, namely BALB/c, SWR, and C3H.SW mice. The resulting antibody responses were of a similar frequency when marrow cells were injected with or without thymocytes. In order to exclude a possible regeneration of a thymic component after irradiation, a transfer experiment was performed in thymectomized, irradiated BALB/c recipients. As can be seen in Table II, the results were similar to those obtained in nonthymectomized mice. Thus, no increase in the response frequencies was observed when 10^8 thymocytes were added to the 2×10^7 bone marrow cells injected.

In contradistinction, the frequency of the immune response to the random copolymer $(Pro^{66}, Gly^{34})_n$ was significantly enhanced when thymocytes were injected together with bone marrow cells. Nevertheless, it seems that the immune response to this antigen was partially thymus independent as the injection of bone marrow cells alone generated a response in about 30% of the animals (Table II).

The response to the tripeptide Pro-Gly-Pro conjugated to a thymus-dependent carrier (ovalbumin) was found to be thymus dependent in BALB/c and C3H.SW mice.

The Role of the Thymus in the Immune Response to Collagen and Gelatin.— The thymus independence of the collagen-like polymer $(Pro\text{-}Gly\text{-}Pro)_n$ suggested that collagen itself may also be a thymus-independent antigen. To test this possibility transfer experiments were performed in which irradiated and cell-repopulated SWR mice were immunized with collagen. This mouse strain was shown to be a high responder to rat tail tendon collagen and to RCM *Ascaris* cuticle collagen (Table I). For comparison, similar transfer experiments were performed with rat tail tendon gelatin, which was obtained by heat denaturation of the native collagen, and is lacking the triple helical conformation possessed by the native molecule. As can be seen in Table III, an efficient immune response to collagens from both rat tail tendon and *Ascaris* cuticle was obtained in the absence of thymocytes. Conversely, the antibody response to gelatin was thymus dependent.

DISCUSSION

The immune response to many synthetic antigens was found to be genetically controlled. Thus, various inbred strains of animal species respond differently to immunization with a given immunogen (8, 18).

In the present study, strain differences were observed in the antibody response to collagen and related polypeptides. Moreover, not all the mouse strains exhibited similar magnitude of response to the collagen-like $(Pro\text{-}Gly\text{-}Pro)_n$ and to the random $(Pro^{66}, Gly^{34})_n$ (Table I). The fact that the same mouse strain was a high responder to the ordered polymer and a low one to the random polymer, or vice versa, suggests that antigenic determinants of different specificities are expressed in these two immunogens.

The responses to collagens from two different species (*Ascaris* cuticle and rat tail tendon) as well as to gelatin were similar in the three mouse strains tested (Table I). Although it could be expected that the antibody response to the collagen-like polymer will resemble that of collagen, BALB/c mice which were low responders to collagen were high responders to $(Pro\text{-}Gly\text{-}Pro)_n$ whereas C3H.SW, the high responders to collagen, exhibited relatively low titers to the ordered polymer.

The main finding of the present study is that a correlation was demonstrated between the three-dimensional conformation of the immunogen and its ability

TABLE II

Thymus-Marrow Cell Cooperation in the Immune Response to $(Pro\text{-}Gly\text{-}Pro)_n$, $(Pro^{66}, Gly^{34})_n$, and Pro-Gly-Pro-ovalbumin

Mouse strain	Immunogen	Assaying antigen	Assayed by*	Cells injected per recipient		
				10^8 thymocytes	2×10^7 marrow cells	10^8 thymocytes and 2×10^7 marrow cells
BALB/c	$(Pro\text{-}Gly\text{-}Pro)_n$	$(Pro\text{-}Gly\text{-}Pro)_n$-RNase	PFC	0‡ (2)	73 (30)	71 (24)
			HA	0 (2)	73 (30)	65 (23)
BALB/c (Tx)	$(Pro\text{-}Gly\text{-}Pro)_n$	$(Pro\text{-}Gly\text{-}Pro)_n$-RNase	PFC	0 (2)	59 (22)	55 (22)
			HA	0 (2)	59 (22)	48 (21)
SWR	$(Pro\text{-}Gly\text{-}Pro)_n$	$(Pro\text{-}Gly\text{-}Pro)_n$-RNase	PFC	15 (13)	53 (15)	64 (11)
			HA	0 (13)	57 (14)	44 (11)
C3H.SW	$(Pro\text{-}Gly\text{-}Pro)_n$	$(Pro\text{-}Gly\text{-}Pro)_n$-RNase	PFC		73 (11)	67 (9)
			HA		55 (11)	44 (9)
BALB/c	$(Pro^{66}, Gly^{34})_n$	$(Pro^{66}, Gly^{34})_n$-RNase	PFC		30 (27)	72 (21)
			HA		42 (19)	70 (19)
C3H.SW	$(Pro^{66}, Gly^{34})_n$	$(Pro^{66}, Gly^{34})_n$-RNase	PFC	11 (9)	32 (22)	67 (18)
			HA	18 (11)	27 (22)	76 (17)
BALB/c	Pro-Gly-Pro-ovalbumin	Pro-Gly-Pro-RNase	PFC		20 (25)	56 (27)
			HA		8 (25)	59 (27)
C3H.SW	Pro-Gly-Pro-ovalbumin	Pro-Gly-Pro-RNase	PFC		5 (19)	56 (9)
			HA		16 (19)	56 (9)

* PFC, hemolytic plaque-forming cell assay. Results higher than 100 plaques per spleen were considered positive. HA, passive microhemagglutination assay. Hemagglutination titer at dilutions greater than 0.25 were considered positive.

‡ Percentage of syngeneic irradiated recipients producing detectable antibody titers. Numbers in parenthesis give the number of mice tested.

TABLE III

Thymus-Bone Marrow Cell Cooperation in the Immune Response to Collagen and Gelatin

Mouse strain	Immunogen and assaying antigen	Assayed by*	Cells injected per recipient		
			10^8 thymocytes	2×10^7 marrow cells	10^8 thymocytes and 2×10^7 marrow cells
SWR	RCM *Ascaris*	PFC	21‡ (28)	74 (38)	70 (23)
	collagen	HA	19 (31)	70 (40)	57 (23)
SWR	Rat tail	PFC	8 (13)	70 (10)	62 (13)
	collagen	HA	23 (30)	77 (35)	83 (30)
SWR	Rat tail	PFC	17 (23)	12 (32)	70 (23)
	gelatin	HA	11 (26)	22 (32)	83 (23)

* PFC, hemolytic plaque-forming cell assay. Results higher than 100 plaques per spleen were considered positive. HA, passive microhemagglutination assay. Hemagglutination titer at dilutions greater than 0.25 were considered positive.

‡ Percentage of syngeneic irradiated recipients producing detectable antibody titers. Numbers in parenthesis give the number of mice tested.

to elicit an efficient immune response in the absence of thymocytes. Two collagens and the collagen-like ordered periodic polymer $(\text{Pro-Gly-Pro})_n$ were shown to be thymus independent (Tables II and III), whereas the denatured product of collagen, gelatin, the random polymer $(\text{Pro}^{66}, \text{Gly}^{34})_n$ and the protein conjugate Pro-Gly-Pro-ovalbumin required the addition of thymocytes for eliciting an effective antibody response.

The thymus independence of $(\text{Pro-Gly-Pro})_n$ was confirmed by the transfer of bone marrow cells into irradiated thymectomized BALB/c recipients (Table II). It should be pointed out that the transfer of 2×10^7 bone marrow cells alone, followed by immunization with the random copolymer $(\text{Pro}^{66}, \text{Gly}^{34})_n$ elicited an immune response in 30–40% of the recipients. This could suggest that the random copolymer contained some stretches possessing the collagen-like conformation. However, the addition of 10^8 thymocytes enhanced the percentage of responses, in contrast to the results with $(\text{Pro-Gly-Pro})_n$ in which no increase was obtained in the number of positive responses in the presence of thymus cells. It thus appears that $(\text{Pro}^{66}, \text{Gly}^{34})_n$ is a thymus-dependent antigen, since we define operationally as thymus-independent antigens those in which the immune response is not enhanced by the addition of thymocytes.

The previously reported thymus-independent antigens were all of relatively high molecular weights. It is of interest that the response to $(\text{Pro-Gly-Pro})_n$ is also thymus independent, even though this periodic ordered polymer possesses a molecular weight of only 6,300. It thus seems that the molecular weight per se does not play a crucial role in defining the need for cellular cooperation.

We have shown previously that macromolecules possessing similar size, shape,

molecular weight, and amino acid composition, and differing only in the optical configuration of amino acid residues in certain unique portions in the molecule, differed greatly in their need for cellular cooperation between T and B cells, in order to produce an efficient immune response (17). In that case the thymus independence of the antibody response was correlated with the slow metabolizability of the immunogens or their component determinants. In the present study it is clearly demonstrated that changes in the steric conformation, unaccompanied by *any* changes in the primary structure of the protein, may lead to dramatic differences in the nature of the triggering of the immune response. These differences may also be correlated with different patterns of metabolism. It should be kept in mind that native collagen, due to its triple helical conformation, is rather resistant to proteolytic digestion by enzymes other than collagenase (34). The latter is probably not present in significant amounts in body fluids, as it is found in various organelles (35). On the other hand, gelatin is very susceptible to proteolytic digestion (36).

It thus seems that a relatively rigid structure may play an important role in the need for cell to cell interaction in order to obtain an efficient antibody formation.

SUMMARY

Several inbred mouse strains were screened for their ability to respond to the ordered periodic collagen-like polymer $(Pro\text{-}Gly\text{-}Pro)_n$, to the random copolymer $(Pro^{66}, Gly^{34})_n$, to the protein conjugate Pro-Gly-Pro-ovalbumin, to rat tail tendon collagen, rat tail tendon gelatin, and to *Ascaris* cuticle collagen. Differences were obtained in the magnitude of the antibody titers towards the above immunogens among the strains tested. The level of the response to the ordered polymer $(Pro\text{-}Gly\text{-}Pro)_n$ was not similar to that towards the random $(Pro^{66}, Gly^{34})_n$, confirming differences in the antigenic determinants of the two immunogens.

The role of the thymus in the immune response to $(Pro\text{-}Gly\text{-}Pro)_n$ and $(Pro^{66}, Gly^{34})_n$ as well as to two collagens and gelatin, was studied in order to find out a possible correlation with the structural features of the immunogens. Heavily irradiated recipients were injected with syngeneic thymocytes, marrow cells, or a mixture of both cell populations and were immunized with the above-mentioned antigens. An efficient immune response to the ordered collagen-like $(Pro\text{-}Gly\text{-}Pro)_n$ was obtained in the absence of transferred thymocytes. The thymus independence of $(Pro\text{-}Gly\text{-}Pro)_n$ was confirmed when thymectomized irradiated mice were used as recipients. In contrast with these results, cooperation between thymus and marrow cells was necessary in order to elicit an immune response to $(Pro^{66}, Gly^{34})_n$. Similarly, the immune response to the triple helical collagen was found to be independent of the thymus, whereas for an effective response to its denatured product, gelatin, thymus cells were required. These findings indicate that a unique three-dimensional structure of immuno-

gens possessing repeating antigenic determinants plays an important role in determining the need for cell to cell interaction in order to elicit an antibody response.

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