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Antibody Formation: Initiation in "Nonresponder" Mice by Macrophage Synthetic Polypeptide RNA

Abstract. *The RNA extracted from normal peritoneal macrophages exposed to a linear, random synthetic polypeptide, $Glu^{60}Ala^{30}Tyr^{10}$, initiated an immune response in C57B1/6J mice, although this strain responds very poorly to the antigen itself. From 10 to 150 micrograms of RNA obtained from mouse, rat, or rabbit macrophages was injected intraperitoneally into recipient mice, and specific antibody was detectable by passive hemagglutination 3 to 4 weeks later. Treatment of the RNA with ribonuclease destroyed its ability to initiate a specific immune response. The RNA contained by weight 0.02 percent of the (specific) antigen. The RNA obtained from cells incubated with a second polypeptide, $Glu^{36}Lys^{24}Ala^{40}$, initiated a response specific for this polymer. This RNA even when incubated in vitro with $Glu^{60}Ala^{30}Tyr^{10}$ failed to initiate antibody formation specific for $Glu^{60}Ala^{30}Tyr^{10}$.*

The role of the macrophage in antibody formation has received much attention. Ribonucleic acid (RNA) extracted from macrophages that had been exposed in vitro to antigen can elicit antibody formation in lymph node cells from nonimmune animals in vitro (*1*) when antigen alone failed to do so. Such RNA is also a potent immunogen in vivo (*2*). We report now on a study of the role of macrophage RNA in a better-defined system, namely, inbred animals and synthetic antigens. The response of inbred mice to structurally simple synthetic polypeptides is genetically determined, both with regard to whether or not the animal can respond ("responder" as opposed to "nonresponder" strains) (*3*), the specificity of the response, and the type and amounts of immunoglobulins produced (*4*). In addition, the animals are free of "background" antibody activity against the antigen.

We used a synthetic, linear, random polymer of the α-amino acids L-glutamic acid, L-alanine, and L-tyrosine, $Glu^{60}Ala^{30}Tyr^{10}$, as antigen (superscripts refer to moles per 100 moles of the amino acid; the molecular weight of the preparation used was 25,000); C57B1/6J mice were the recipients of the RNA. The antibody response of this strain to $Glu^{60}Ala^{30}Tyr^{10}$ is normally very poor. The animals fail to respond to 0.01 μg to 1 mg of the polymer in saline, and respond poorly even to polymer in Freund's complete adjuvant; mean passive hemagglutination titers on day 14 were 1:16, and they declined rapidly thereafter. This is in contrast to the excellent antibody response (100 to 800 μg of antibody nitrogen per milliliter of serum) of other strains (*3*). Upon restimulation, the C57B1/6J mice fail to show an increased response, and in fact become completely unresponsive. With pertussis vaccine as adjuvant an antibody response—first detectable 10 days after immunization—is produced; the biological properties of the antibody produced, which is also transient and cannot be restimulated, indicate that it is the murine equivalent of immunoglobulin E (IgE) (*5*).

The techniques used for obtaining peritoneal exudates that contain large amounts of macrophages, incubation with antigen, and extraction of RNA with phenol have been described (*6*). Antibody was determined by passive hemagglutination (*7*). Macrophages of other inbred mouse strains and of heterologous species were examined as sources of active RNA (Table 1). Antibody was detected in the serums of a significant number of mice 2 or 3 weeks after the intraperitoneal injection of 25 to 100 μg of macrophage RNA. Although the titers were low, rarely exceeding 1:16, they were reproducible and specific. The titrations [micro-Takatsy method (*3*)] yield values which are consistently three to five doubling dilutions lower than those obtained with a tube method.

The macrophage donors for groups 1 and 2 were nonimmunized mice of strains that produce antibodies to the

Table 1. Antibody to $Glu^{60}Ala^{30}Tyr^{10}$ (GAT) in primed and unprimed C57B1/6J mice subsequently treated with RNA extracted from homologous and heterologous normal macrophages that had been incubated with GAT. Priming consisted of injecting (intraperitoneally) the recipient mice with (A) 1 mg of GAT in 0.15*M* NaCl plus 500 μg of pertussis vaccine; (B) 1 mg of GAT in 0.15*M* NaCl; or (C) 0.15*M* NaCl. Three months later RNA in sterile tris buffer, *p*H 7.2, was injected intraperitoneally. Antibody titers were determined by the passive hemagglutination of sheep red cells coupled with polymer by bis-diazobenzidine, by serums which had been absorbed with sheep red cells. Results are the ratio of the number responding to the number tested.

Group	Donor macrophages		Treatment of recipient mice		Antibody response on day:				
	Source	Polymer added per 10^9 cells (mg)	Priming	RNA (μg)	8	14	21	28	35
1	C3H/HeJ mice	10	A	35, 50*	5/6	5/6	5/6	0/2	
2	DBA/2 mice	50	A	25		1/5	1/5		1/4
			B	25		1/6	1/5		1/5
			C	25		2/9	4/7		1/7
3	Rat	50	A	56		3/5	2/4		0/4
			B	56		2/5	3/5		2/5
4	Rabbit	45	A	100		1/3	1/2		2/2
			B	100		2/5	4/4		2/4
			C	100		1/4	3/4		3/3

* Two separate preparations of RNA were used.

Table 2. Effect of amount of $Glu^{60}Ala^{30}Tyr^{10}$ polymer (GAT) incubated with 10^9 normal rabbit macrophages on the ability of the RNA extracted from these cells to initiate production of antibody to GAT in unprimed C57B1/6J recipients of the RNA. The RNA was injected intraperitoneally in sterile tris buffer, *p*H 7.2. The response was determined by passive hemagglutination of sheep red cells coupled to GAT with bis-diazobenzidine. The results represent the ratio of the number of mice responding to the number tested.

Cell preparation	Polymer per 10^9 donor macrophages (mg)	Recipient mice: RNA per mouse (μg)	Recipient mice: Response on day 28
1	0.05	80	0/8
	.5	85	0/6
2*	.01	11.5	6/8
	.01	145	6/9
	1.0	10	3/8
	1.0	100	9/9
3	1.0	50	3/5
	1.0	50 + ribonuclease†	0/5
4	30	10.5	2/6
	30	105	4/8
	30	105 + ribonuclease†	0/8
5	45	100	9/10

* These cells were incubated with a C^{14}-GAT preparation. † Ribonuclease A (Worthington) in tris buffer, *p*H 7.2, was added in the ratio of 1:16 (by weight); the mixture was kept at 37°C for 15 minutes.

polymer (*4*). When macrophages of rats (group 3) or rabbits (group 4) were incubated in vitro with the polymer, the RNA extracted was immunogenic. Prior treatment of the recipient mice with polymer alone, or with pertussis vaccine and polymer apparently has little effect on the response of the animals to the antigenic stimulation induced by the RNA.

Since the more readily available rabbit macrophages proved suitable and since the recipient mice which had received no prior treatment were also suitable, we used this combination. A series of experiments were designed to determine the response of macrophages —in terms of the immunogenicity of the extracted RNA—to varying doses of the polymer, and the response of the recipient mice to varying doses of RNA. There is considerable variability between different cell preparations, possibly due to varying degrees of degradation of the RNA in preparation or upon storage (Table 2). For example, cell preparation 1 was inactive when 80 to 85 μg of RNA per mouse was injected; but preparation 2, in which the cells had been exposed to similar amounts of polymer was highly active when 10 to 145 μg of RNA was injected.

Thus, varying proportions of antigen and cells produce an active RNA. Further data are needed to establish dose-response curves, not only for the polymer-macrophage step but also for the RNA-antibody step. For groups 3 and 4 immunogenicity was destroyed by treatment with ribonuclease. Although the ratio of enzyme to substrate was quite high (1:16), the species of RNA responsible for the late response is relatively resistant at lower ratios, and the inactivation of the RNA is dependent upon the enzymatic properties of ribonuclease and not upon nonspecific combination with the RNA (*8*).

The RNA extracts were examined for antigen by the precipitin reaction and complement fixation, and also by exposing the cells to C^{14}-labeled-$Glu^{60}Ala^{30}Tyr^{10}$ (Table 2, group 2). The RNA preparation from cells exposed to 1 mg of labeled polymer (Table 1, group 2, treatment B) contained radioactivity equivalent to 0.02 percent, by weight, of polymer. This is comparable to the results found with bacteriophage T2 (*8*). No radioactivity above background was detected in RNA from cells exposed to 0.01 mg of polymer (group 2, treatment A); thus the recipients of the RNA in this group received less than 0.001 μg of polymer (recipients of 11.5 μg RNA) to 0.015 μg polymer (recipients of 145 μg of RNA). The state and relevance of the antigen in the RNA preparation remain to be determined.

A final series of experiments (Table 3) illustrates the specificity of the response, provides evidence against the concept that the RNA serves simply as an adjuvant, and introduces data that show a good antibody response to a different polymer ($Glu^{36}Lys^{24}Ala^{40}$) with RNA obtained after "processing" by macrophages. The cell preparation used was the same one used for group 4, Table 2. The recipients received either 10 or 100 μg of RNA alone, (groups 1 and 2) or 100 μg of RNA and 0.01 to 1.0 μg of $Glu^{60}Ala^{30}Tyr^{10}$ (groups 3 to 5). The serums obtained from 7 to 40 days after injection of RNA were studied for antibody to $Glu^{60}Ala^{30}Tyr^{10}$ and antibody to $Glu^{36}Lys^{24}Ala^{40}$. At no time did any of the animals produce antibody to $Glu^{60}Ala^{30}Tyr^{10}$, although between 21 and 35 days all were positive for antibody to $Glu^{36}Lys^{24}Ala^{40}$.

Our data also demonstrate that the failure of C57B1/6J mice to respond well, if at all to $Glu^{60}Ala^{30}Tyr^{10}$ is not due to an overloading with antigen, for a 0.01-μg dose failed to immunize these animals.

Table 3. Specificity of formation of antibody in unprimed C57B1/6J mice. Normal rabbit macrophages were incubated (15 minutes at 37°C) with $Glu^{36}Lys^{24}Ala^{40}$ (45 mg per 10^9 cells). The RNA was extracted from these cells and suspended in sterile tris buffer, *p*H 7.2. Portions of the RNA were incubated in vitro with $Glu^{60}Ala^{30}Tyr^{10}$ (GAT), as indicated, for 15 minutes at 37°C. The mixture was injected intraperitoneally on day zero. Antibody titers were determined by passive hemagglutination, with the use of GAT coupled to sheep red cells by bis-diazobenzidine for antibodies to GAT and tanned sheep cells coated with $Glu^{36}Lys^{24}Ala^{40}$ (GLA) for antibodies specific for $Glu^{36}Lys^{24}Ala^{40}$. Results are expressed as the ratio of the number responding to the number tested.

Group	Material injected: RNA (μg)	Material injected: Polymer (μg)	Antibody response on day 28 to: GAT	Antibody response on day 28 to: GLA
1	10	None	0/8	8/8
2	100	None	0/8	8/8
3	100	0.01	0/7	7/7
4	100	.1	0/7	7/7
5	100	1.0	0/7	7/7

Thus, when normal macrophages from several species are incubated with synthetic polypeptides, the RNA subsequently extracted can elicit antibody to the polymer in normal "nonresponder" mice. The response is specific, and is dependent upon the integrity of the RNA. Although antigen is present in the extracts the responses obtained cannot be due merely to the presence of the polymer alone.

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