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Immune Response in vitro: Independence of "Activated" Lymphoid Cells

Abstract. *Antibody formation against sheep erythrocytes by mouse spleen cells in vitro requires interactions among antigen-treated macrophages and lymphoid cells in cell clusters for only a finite time. During this critical period of interaction, lymphoid cells become "activated" and thereafter can develop into antibody-producing cells independently of native antigen, macrophages, and cell clusters.*

The development of an immune response to sheep erythrocytes in vitro requires both macrophages and lymphoid cells. Macrophages, after exposure to antigen, stimulate lymphoid cells to develop into antibody-producing cells (*1*). The "stimulation" probably occurs through cell interactions in clusters. The continued integrity of these cell clusters is thought to be essential for the development of a maximum plaque-forming cell (PFC) response to sheep erythrocytes (*2*). We present data to support the concept that interactions among antigen-treated macrophages and lymphoid cells in cell clusters are essential only for a finite time during which the lymphoid cells become "activated." Thereafter, "activated" lymphoid cells destined to replicate and develop into antibody-producing cells are independent of macrophages, cell clusters, and native antigen.

The culture reagents, culture conditions, and cell population separation procedures used have been described (*1*, *3*). Single cell suspensions from spleens of unimmunized, 6- to 8-month-old C57Bl/6N mice were separated into macrophage and lymphoid cell populations (*4*). Macrophages (10^6 per dish) were incubated with 10^7 sheep erythrocytes (SRBC) for 60 minutes. After unbound erythrocytes were removed, the cultures were reconstituted with 10^7 lymphoid cells. Culture dishes were incubated for specified periods on a slowly rocking platform to facilitate both formation of cell clusters and cell interactions. Transfer of dishes to a stationary platform stopped formation of new cell clusters. Cell interactions were interrupted by transfer of the dishes to the stationary platform after dispersion of cell clusters by vigorous pipetting. After 4 days of incubation both clusters of cells and individual plaque-forming cells that release IgM antibodies were enumerated by a modification of the hemolytic plaque technique (*5*).

In this culture system, maximum PFC responses developed in cultures incubated on the rocking platform for 4 days (control maximum response, Table 1). After 6 hours of incubation cell clusters were visible with phase microscopy in these cultures and persisted throughout the 4 days. Cell-cluster plaques contained three to four PFC. In contrast, stimulated cultures incubated on the stationary platform for 4 days developed only minimum responses (control minimum response) similar to responses in unstimulated cultures. Rare, small cell clusters were visible, and cell-cluster plaques contained only about one PFC.

The conditions necessary for "activation" of lymphoid cells were determined by measuring effects on the PFC response, which result from manipulations on the cells in the cultures after specified periods of incubation. Transfer of the cultures from the rocking to the stationary platform after 48 hours of incubation with or without dispersion of cell clusters did not reduce the PFC response. Cultures transferred at 6 hours with or without dispersion of cell clusters developed only minimum responses, whereas cultures transferred at 24 hours developed responses that were one-third to one-half those of the control maximum responses. In cultures transferred without dispersion, cell clusters persisted and cell-cluster plaques had two to six PFC (Table 1). In contrast, in cultures transferred after dispersion, only rarely did cell clusters re-form, and cell-

Table 1. Plaque-forming cell responses of spleen cells to sheep erythrocytes under differing conditions of culture.

Culture conditions	PFC/10^6 recovered cells*	PFC per cell-cluster plaque†	Visible cell clusters	
			Number	Size
Control, 96 hours rocking				
+SRBC (control maximum response)	861	3.41	Many	Medium
−SRBC	66	—	Rare	Large
Control, 96 hours stationary				
+SRBC (control maximum response)	68	1.02	Rare	Small
−SRBC	40	—	Rare	Small
Hours rocking before transfer to stationary platform				
6	74	5.06	Rare	Small
24	380	5.92	Few	Large
48	890	2.33	Many	Medium
Hours rocking before dispersion of cell clusters and transfer to stationary platform‡				
6	60	1.70	Rare	Small
24	446	1.04	Rare	Small
48	1017	1.05	Rare	Small

* Values from a representative experiment. † Values approximating 1.0 indicate PFC are not in clusters. Values greater than 1.0 indicate PFC are in clusters. Each value represents the average of at least ten duplicate determinations from each type of culture. The minus size indicates less than ten determinations. ‡ Dispersion of cell clusters and returning cultures to the rocking platform at 6, 24, and 48 hours had no effect on the PFC response; the data (including average PFC per cell-cluster plaque and the number and size of visible cell clusters) were similar to the control maximum response and are not known.

cluster plaques had only one PFC. Controls demonstrated that dispersion of cell clusters and return of the cultures to the rocking platform was without deleterious effect on the PFC response (footnote, Table 1). Thus, transfer to the stationary platform with or without dispersion of cell clusters before 48 hours of incubation stopped the "activation" process at that point, causing a reduced PFC response. Further, the "activation" process appeared complete by 48 hours of incubation on the rocking platform, a time when very few PFC were detected.

The only antigen in this culture system was that associated with macrophages. The duration of the interactions among macrophages and lymphoid cells required for "activation" of the latter was estimated by depleting macrophages from cultures after 24 or 48 hours of incubation (Table 2). Nonadherent cells and cell clusters were transferred to new culture dishes, cell clusters were dispersed, and the macrophages were separated from lymphoid cells as described (*1*). Less than 0.01 percent of the nonadhering cells, after three incubation periods during which macrophages attached to the culture dishes, could be unequivocally identified as phagocytic cells on the basis of uptake of neutral red or polystyrene latex beads. These lymphoid cells were then incubated on either the rocking or stationary platform.

There was no difference between the PFC responses in the control cultures and either the stationary or rocking cultures depleted of macrophages at 48 hours (Table 2). In all depleted cultures, visible cell clusters were rare; PFC appeared to exist singly. It is possible that PFC may have existed in clusters with one or two "other" specific cells that were not detected as PFC. However, in the cell cluster assay, the rare clusters seen did not form plaques, while the plaques themselves contained only one visible nucleated cell. Thus the depletion process appeared to remove a factor required for formation of cell clusters containing specific PFC, presumably the antigen-bearing macrophage.

The PFC response was reduced 50 percent in cultures incubated on the stationary platform after depletion of macrophages at 24 hours. However, there was no reduction in PFC response in cultures returned to the rocking platform after depletion at this time. The most interesting observation was that the PFC response was comparable to the control maximum response if, after depletion of macrophages at 24 hours, the cultures were incubated on the rocking platform for an additional 24 hours before transfer of the lymphoid cells to the stationary platform with dispersion of the rare, small cell clusters.

Table 2. Plaque-forming cell response of "activated" lymphoid cells under differing conditions of culture after depletion of macrophages.

Culture conditions	PFC/10^6 recovered cells*	PFC per cell-cluster plaque*	Visible cell clusters	
			Number	Size
Control, 96 hours rocking	979	3.41	Many	Medium
48 hours rocking, deplete macrophages †, then incubate				
Rocking	987	1.10	Rare	Small
Stationary	1063	1.06	Rare	Small
24 hours rocking, deplete macrophages, then incubate				
Rocking	933	1.04	Rare	Small
Stationary	377	1.09	Rare	Small
Rocking for next 24 hours, then transfer to stationary	899	1.03	Rare	Small

* As in the legend of Table 1. † Macrophages were depleted by dispersion of cell clusters and by allowing macrophages to adhere to culture dishes (*1*). Prior to depletion, the ratio of macrophages to lymphoid cells was 1 to 10; after depletion, it was less than 1 to 10,000.

These data suggest that "activation" is at least a two-step cell interaction process involving (i) a macrophage-dependent phase complete in about 24 hours followed by (ii) a macrophage-independent phase complete in the next 24 hours. The possibility that the macrophage-independent phase involves interactions among a few remaining antigen-bearing phagocytic cells and lymphoid cells cannot be disregarded, but if this were the case, one would expect these phagocytic cells to be foci for formation of cell clusters. However, cell clusters containing PFC were not detected in cultures depleted of macrophages. The data rather suggests that the macrophage-independent phase may involve transient interactions among "activated" and nonactivated lymphoid cells. In view of the observations by others, macrophages after uptake of antigens may interact with and activate intermediate cells (possibly thymus-derived) that do not become PFC (macrophage-dependent phase). These cells then interact with and "activate" cells (possibly bone marrow-derived) destined to become antibody producers (macrophage-independent phase) (*6*).

The data presented also demonstrate that sufficient lymphoid cells must be "activated" by 48 hours, when few PFC are detected, to develop the 4-day maximum responses observed. No or only a very few cells can be recruited for the response by "activated" lymphoid cells or activated intermediate cells in stationary cultures after dispersion of cell clusters or depletion of macrophages at 48 hours, since cell interactions are minimum and cell clusters do not re-form. Yet, maximum responses develop. Independent "activated" lymphoid cells do, therefore, develop by cell division or differentiation, or both, into antibody-forming cells in the absence of macrophages, native antigen, and cell clusters.

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4. The separation procedure yielded about 10^6 macrophages per dish from a spleen cell suspension containing 1 to 2×10^7 cell/ml. Macrophages were operationally defined as cells adhering to the culture dishes during the separation procedure; about 98 percent of these adhering cells were phagocytic by criteria of polystyrene latex bead (1.1 μ) and neutral red uptake. Lymphoid cells were those that did not adhere to the culture dishes; less than 2 percent engulfed polystyrene latex beads or neutral red. Separated macrophages and lymphoid cells incubated with 10^7 SRBC for 4 days did not develop a response greater than background.
5. N. K. Jerne and A. A. Nordin, *Science* **140**, 405 (1963); P. H. Plotz, N. Talal, R. Asofsky, *J. Immunol.* **100**, 744 (1968) The response was determined from duplicate or triplicate assay slides for each experimental dish. The number of plaque-forming cells per cell-cluster plaque was estimated as follows. Cells were gently aspirated from the culture dish with a 1.0-ml pipette; 0.1-ml portions were added directly to tubes containing thoroughly mixed agarose and sheep erythrocytes; the contents of the tube were poured onto prepared slides with minimum agitation (assay for cell-cluster plaques). The cells remaining in the pipette were diluted 1:5 in Hanks' balanced salt solution; cell clusters were dispersed by vigorous agitation. The resulting single-cell suspension was then assayed for individual plaque-forming cells. Results were expressed per 10^6 recovered cells.

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Polynucleotide Sequence Analysis by Sequential Base Elimination: 3′-Terminus of Phage Qβ RNA

Abstract. *The nucleotide sequence of a 3′-terminal fragment obtained by ribonuclease T_1 hydrolysis of the ribonucleic acid from bacteriophage Qβ has been determined by an improved method of sequence analysis which involves sequential removal of bases by periodate oxidation and beta-elimination. The results obtained from ten such oxidation-elimination cycles and from the alkaline hydrolysis of the remaining oligonucleotide indicate that the first 16 nucleotides at the 3′-terminus of this ribonucleic acid have the sequence:*
-G-C-C-C-U-C-U-C-U-C-C-U-C-C-C-A.

The initial phase in the sequence analysis of RNA molecules (*1*) of high molecular weight has involved the development of methods for the investigation of the primary structures near their terminals. Although these methods are expected to produce only a limited amount of sequence information relative to that contained in the whole molecule, it is considered that such information will be of some value in studies on structure-function relations in RNA. In the case of viral ribonucleic acids, the interest in terminal structures results from the expectation that they will be found to contain specific nucleotide sequences which signal for the initiation and termination of both the replication and translation of these molecules. The terminal sequence -G(C_9U_4)C-A of the RNA from the bacteriophage Qβ has already been shown to differ from the corresponding sequences in the phages f2 and MS2, and it has been suggested that this difference in sequence may constitute the molecular basis of the observed template specificities displayed by the Qβ and MS2 polymerases (*2*).

For the specification of the actual order of the 14 pyrimidines at the Qβ terminus we have studied further the periodate-beta-elimination method that was developed for the determination of the terminal sequence -G-U-U-A-C-C-A-C-C-C-A of the f2 RNA (*3*). In this method the bases are removed sequentially from the 3′ end of the polynucleotide by cycles of operations consisting of (i) periodate oxidation of the terminal glycol group; (ii) treatment of the resulting dialdehyde with a primary amine to effect the removal of the terminal base by a beta-elimination reaction; and (iii) the removal of the terminal phosphate group, so produced, by phosphatase to regenerate the polynucleotide in a form ready for the next cycle. Previously we had used a cyclohexylamine solution buffered with di-*n*-propylmalonic acid for the beta-elimination step, because this mixture gave essentially quantitative yields when applied to the cleavage of small oligonucleotides. However, the results of experiments with this amine solution and larger oligonucleotides have indicated that, in these cases, the yields of the beta-elimination reaction fall somewhat short of the desired 100 percent. A new amine mixture containing cyclohexylamine and tetramethylglycinamide is now being used for this reaction, because, in experiments with both dinucleotides and hexanucleotides, this solution has been found to effect over 97 percent removal of the oxidized terminal nucleosides. The tetramethylglycinamide also serves another purpose in that it is used as the buffer for the subsequent enzymatic dephosphorylation step in each cycle. A second major modification in this method of sequence analysis resulted from the observation that the DEAE-Sephadex chromatography used to separate the liberated base from the polynucleotide fragment resulted in the incomplete recovery of the fragment in each cycle. This problem has now been solved by substituting, for the chromatographic matrix, DEAE-cellulose from which the recoveries are rapid and essentially quantitative.

In a typical oxidation-elimination cycle the polynucleotide (10 to 100 nmole) in water (250 μl) is treated with EDTA (free acid, 2.0 mg) to inactivate any phosphatase which is carried over from the previous cycle. The mixture is allowed to stand for 1 hour at 20°C with occasional shaking and then the amine solution (1*M* cyclohexylamine—2*M* *N,N,N′,N′*-tetramethylglycinamide-HCl; 100 μl) is added. When the EDTA is completely dissolved, 0.2*M* sodium periodate (50 μl) is added, and the mixture is kept at 45°C for 2 hours. The solution is then treated with 0.4*M* ribose (50 μl) to remove any unreacted periodate. After incubation of the solution for 15 minutes at 20°C, water (500 μl) and alkaline phosphatase [2 units (*4*) in 100 μl of water] are added (final *p*H = 8.0 at 37°C). The mixture is incubated at 37°C for 2 hours and then adjusted to *p*H 9.0 by the addition of 2*M* ammonium hydroxide (200 μl), and finally applied to a DEAE-cellulose column (Whatman DE23, HCO_3^- form, *p*H 9.0; 0.5 by 50 cm). The column is eluted with water to recover the liberated base and then treated with a linear gradient of triethylammonium bicarbonate (200 ml, 0.1 to 1.0*M*) at a flow rate of 15 ml/hour to recover the polynucleotide fragment. The combined fractions containing the fragment are then concentrated to dryness ready for the next cycle.

The 3′-terminal fragment of Qβ RNA was isolated by the method previously described (*5*). In this method the RNA is first digested with ribonuclease T_1 and then treated with sodium periodate. The oxidized terminal fragment is selectively absorbed on an aminoethyl-cellulose column and subsequently recovered from the column by treatment with *n*-propylammonium bicarbonate. This treatment releases the fragment by a beta-elimination reaction, and the fragment is obtained in a form that lacks its terminal nucleoside. The fragment from Qβ RNA obtained in this way had the composition (C_9U_4)Cp (*2*). This polynucleotide was dephosphorylated, and the product was submitted to ten cycles of periodate oxidation-beta-elimination. The order of the bases released from the 3′ end was: C,C,C,U,C,C,U,C,U,C. The identity of the base released in each degradation cycle was determined by its position of elution from the DEAE-cellulose column and by its ultraviolet spectrum. In a number of cases the entire water eluate from the column was analyzed on a small column of Dowex 50 × 8 (200 to 400 mesh, 0.4 by 60 cm) (*6*) to confirm that only one base was released at each degradation cycle. The recovery of the base from each cycle was more than 80 percent, based on the amount of oligonucleotide introduced into the cycle. For example, in cycle five, 4.25 O.D.U. at 260 nm of oligonucleotide gave 0.32 O.D.U. at 265 nm of cytosine.

The remaining part of the sequence of the terminal fragment was determined by the alkaline hydrolysis of the oligonucleotide that remained after the ten degradation cycles. This oligonucleotide (0.45 O.D.U. at 260 nm) was hydro-