IMPORTANCE OF THE DENSITY OF THE SPLEEN CELL SUSPENSION IN CULTURE FOR DEVELOPMENT OF THE IMMUNE RESPONSE

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The number of antibody-forming cells (AFCs) formed at the end of incubation was shown to depend on the number of cells of a spleen suspension used to induce the primary immune response in vitro. With an increase in the "surface density" of the cells (the number of cells per cm^2 of the bottom of the incubation vessel) the number of AFCs formed was reduced by 10-100 times although the total number of cells which survived at the end of incubation was not reduced or was only very slightly reduced. The effect was observed when either sheep's red cells or water-soluble antigen extracted from them was used as the antigen. It was independent of a deficiency of antigen or of nutritive substances and it was accompanied by a parallel overall decrease in the incorporation of thymidine-H^3 into the cells in culture.

KEY WORDS: immune response in vitro; immunocompetent cells; intercellular interaction.

The appearance of methods of inducing a primary immune response in vitro [3, 6] has broadened the outlook for analysis of this process. In particular, these methods are widely used to study the role of interaction between cells in the induction of the immune process [1, 2].

The object of this investigation was to study the effect of the number of spleen cells taken for culture on the development of the immune response in vitro.

EXPERIMENTAL METHOD

Experiments were carried out on C57BL/6 mice. Induction of antibody formation in vitro was studied by the method of Mishell and Dutton [6], modified by Click et al. [3] and with minor modifications by the writers [1].

The prepared suspension of spleen cells [1] was poured together with antigen into silicone-treated (using "Antifoam Silane") penicillin flasks, 22 mm in diameter, filled with a gas mixture (5% CO_2, 10% O_2, 85% N_2), sealed with rubber stoppers, and incubated for 1-4 days at 37°C. Sterile sheep's red cells (SRBCs) or water-soluble antigen of sheep's red cells (WSA), isolated by the method of Seman et al. [7], were used as the antigen. The number of IgM-antibody-forming cells (AFCs) in each culture was determined by the method of Jerne and Nordin [5] and the number of living cells was determined by staining with a mixture of eosin and Trypan Blue.

EXPERIMENTAL RESULTS

The number of AFCs determined by Jerne's method in the original suspensions of spleen cells from normal C57BL/6 mice was 0.8-1.5 per 10^6 living cells. After incubation of the cells for 3-4 days, both in the presence of SRBC and in the presence of WSA isolated from them, the number of AFCs increased to 500-1500 per 10^6 living cells.

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TABLE 1. Effect of Certain Factors on AFC Formation in Cultures with Different Numbers of Cells

Expt.	Factors studied	Number of AFCs per 10^6 living cells in cultures containing different numbers of cells		
		$5 \cdot 10^6$	$10 \cdot 10^6$	$20 \cdot 10^6$
3—19	Number of cells taken for culture (antigen SRBC)	672±255	439±52	9±6,3
3—30	Number of cells taken for culture (antigen WSA)	818±363	197±75	7±1,3
3—35	Antigen concentration (antigen WSA) (in μg):			
	5	296±70	152±56	17±6,4
	50	677±202	366±74	71±47
	100	584±120	296±33	35±12
3—36	Concentration of 2-mercaptoethanol (in M):			
	$1,7 \cdot 10^{-5}$	180±25		23±11
	$5,0 \cdot 10^{-5}$	163±42		33±5,5
	$15,0 \cdot 10^{-5}$	273±21		36±6,9
3—37	Concentration of calf embryonic serum (in %):			
	10	469±96	97±23	5±1,5
	20	304±61	97±28	9±0,8
	30	383±157	44±2,0	5±2,7
3—37	Incubation medium (amino acids and other additives to Eagle's minimal medium):			
	normal dose	469±96	97±23	5±1,5
	three times normal dose	840±245	391±72	23±9,5

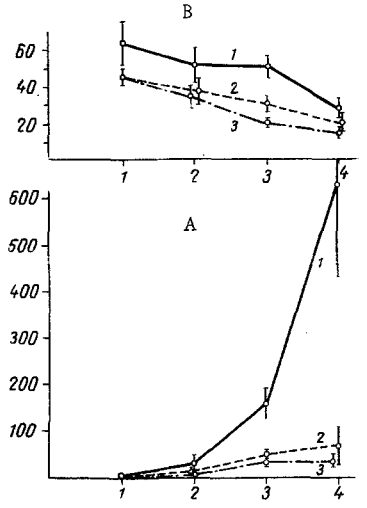

Fig. 1

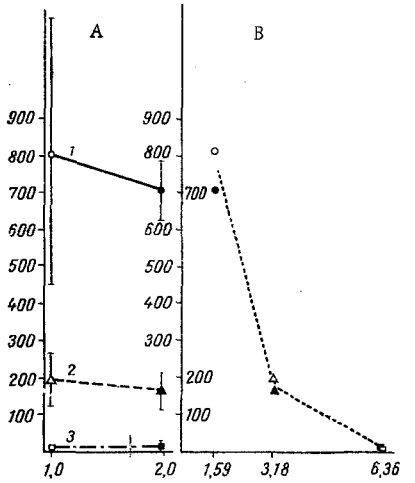

Fig. 2

Fig. 1. Development of immune process (A) and survival rate of cells (B) in cultures containing different numbers of cells: 1) $5 \cdot 10^6$, 2) $10 \cdot 10^6$, 3) $20 \cdot 10^6$ cells per flask. Abscissa, time after beginning of cultivation (in days); ordinate: in A) number of AFCs per 10^6 living cells, in B) percentage of surviving cells.

Fig. 2. Effect of sample volume (A) and "surface density" of cells (B) on intensity of immune response: 1) $5 \cdot 10^6$, 2) $10 \cdot 10^6$, 3) $20 \cdot 10^6$ cells per flask. Abscissa: in A) volume of samples (in ml), in B) "surface density" (number of cells per cm^2 bottom of flask $\cdot 10^6$); ordinate, number of AFCs per 10^6 living cells.

Intensive formation of AFCs was observed only if a certain number of cells was taken for culture. If the number of cells in the sample was increased by two or four times there was a sharp decrease in the number of AFCs detectable at the end of incubation. This decrease was observed in all cases although it varied in degree. In some experiments the number of AFCs was reduced by 4-10 times, but in most cases the decrease was greater – by 50-100 times (Table 1, experiments 3-19 and 3-30). It is interesting to note

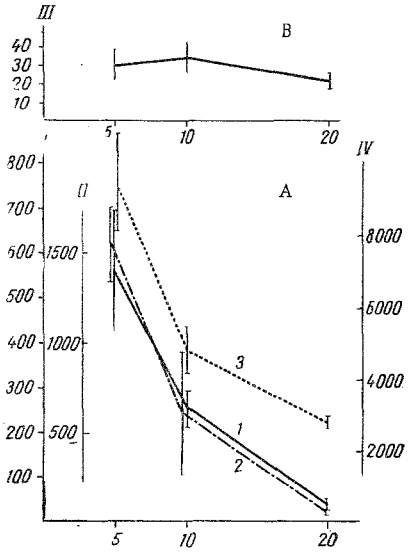

Fig. 3. Intensity of immune response and incorporation of thymidine-H^3 and glycine-C^{14} (A) and also survival rate of cells (B) as functions of density of incubated cell suspension: 1) number of AFCs formed per 10^6 living cells; 2) incorporation of thymidine-H^3; 3) incorporation of glycine-C^{14}. Abscissa: number of cells in sample $\cdot 10^6$; ordinate: I) number of AFCs per 10^6 living cells; II) incorporation of thymidine-H^3 (in counts/min/10^6 living cells); III) percentage of surviving cells; IV) incorporation of glycine-C^{14} (in counts/min/10^6 living cells).

that if WSA was used the decrease in the number of AFCs usually began to appear with fewer cells in the culture than in the experiments with SRBC.

Jerne's direct method, of course, detects only IgM-AFCs. It was necessary to find out whether the observed decrease in the number of IgM-AFCs was connected with the switch of the cells over to synthesis of IgG antibodies. Comparison of the number of IgM-AFCs and IgG-AFCs (Jerne's indirect method with rabbit serum against mouse immunoglobulins) in cultures containing different numbers of cells showed that no such change took place in antibody synthesis.

The decrease in the number of AFCs with an increase in the number of cells taken for culture was not due to the rapid death of the cells in those cultures. Usually the percentage of cells remaining alive by the end of incubation was the same in cultures with a larger number of cells or it was only 1.5-2 times less than in cultures with a small number of cells. Moreover, it must be remembered that the intensity of the immune response was assessed and expressed relative to living cells.

It was interesting to compare the dynamics of the increase in the number of AFCs during development of the immune process in cultures containing different numbers of cells. It will be clear from Fig. 1 that, with the optimal number of cells in the culture ($5 \cdot 10^6$) the number of AFCs increased rapidly along an exponential curve.

In cultures in which the initial number of cells was $10 \cdot 10^6$ and $20 \cdot 10^6$ the increase in the number of AFCs was slow and slight. The first question to arise is: Was the phenomenon observed connected with the concentration of the cells (in 1 ml medium) or with their "surface density" (the number of cells per cm^2 of the flask bottom)? Comparison of the relationship between AFC formation and the volume of the samples or the number of cells taken per flask showed the decisive role of the "surface density." It will be clear from Fig. 2 that the number of AFCs fell sharply with an increase in the "surface density" but was almost independent of the sample volume.

The dependence of AFC formation during an increase in the density of the cultures on the quantity of antigen taken to reduce the immune response in vitro was studied in a series of experiments. The results in Table 1 show that an increase in the antigen concentration did not greatly affect the AFC population although the absolute level of the immune response increased under these conditions (Table 1, experiment 3-35). An increase in the concentration of such important elements as embryonic calf serum and 2-mercaptoethanol in the incubation medium likewise had no appreciable effect on the AFC population (Table 1, experiments 3-36 and 3-37).

It was also necessary to discover whether the observed effect was connected with a deficiency of nutritive substances in the cultures with a large number of cells. To test this hypothesis a medium was prepared in which three times the normal dose of essential and nonessential amino acids, vitamins, nucleic acid precursors, glutamine, sodium pyruvate, glucose, and insulin was present. The absolute number of AFCs in the enriched medium was several times greater than in the normal medium. However, even in the enriched medium, in cultures containing $20 \cdot 10^6$ cells the number of AFCs was almost 40 times less than in cultures containing $5 \cdot 10^6$ cells (Table 1, experiment 3-37).

To study the biochemical characteristics of cultures with different cell densities, the incorporation of thymidine-H^3 and glycine-C^{14} was studied (Fig. 3). Thymidine-H^3 was added at the beginning of incubation in a dose of 0.1 μCi/ml, whereas glycine-C^{14} (1 μCi/ml) was added to the samples 8 h before the end of incubation. As Fig. 3 shows, with an increase in the number of cells in the culture the number of AFCs

and incorporation of thymidine-H^3 showed a parallel decrease but the rate of incorporation of glycine-C^{14} decreased more slowly.

The experiments described above show conclusively that an increase in the number of cells in the sample taken leads to a sharp decrease in the number of AFCs formed during incubation. This phenomenon is not due to deficiency of antigen, for even a 20-fold increase in the amount of antigen did not abolish the effect. It is only a little dependent on the volume of the nutrient medium (Fig. 2) and it is unconnected with a deficiency of amino acids or of other essential substances for cell metabolism in the medium (Fig. 3).

The leading role in the decrease in AFC formation is played by an increase in the density of cells settling on the bottom of the flask during incubation and the consequent depression of cell proliferation.

The results thus indicate that the development of the immune process depends not merely on the ratio between cells of different types (B and T lymphocytes, A cells), but also on the "surface density" of these cells. This conclusion is confirmed by data obtained by the writers previously [2].

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