Recent studies on bacteria (*6*), phyto- and zooplankton (*6*, *13*), and "coral" reef organisms (*17*) have shown a ratio of C-odd to C-even *n*-paraffins close to unity, and therefore these organisms appear to be better candidates than higher plants for biological precursors of petroleum of marine origin.

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References and Notes

1. J. Oró, D. W. Nooner, S. A. Wikström, in preparation.
2. R. Ryhage, *Anal. Chem.* **36**, 759 (1964).
3. A. H. Warth, *The Chemistry and Technology of Waxes* (Reinhold, New York, 1947); H. J. Deuel, Jr., *The Lipids and Their Biochemistry* (Interscience, New York, 1951–1957), vol. 1, pp. 305–404, and vol. 3, pp. 319–359; E. C. Horning and W. J. A. Van den Heuvel, *Ann. Rev. Biochem.* **32**, 709 (1963).
4. These ratios of peak heights should be taken only as a first approximation since they may vary, depending on the degree of resolution and other features of the chromatographic separation. Ratios higher than 8 have been obtained for the whole plant using capillary columns.
5. J. D. Waldron, D. S. Govers, A. C. Chibnall, S. H. Piper, *Biochem. J.* **78**, 435 (1961); G. Eglinton and R. J. Hamilton, in *Chemical Plant Taxonomy*, T. Swain, Ed. (Academic Press, London, 1963), p. 187.
6. J. Oró, D. W. Nooner, S. A. Wikström, unpublished data.
7. F.B. Power and A. H. Salway, *J. Chem. Soc.* **97**, 231 (1910); H. Rogerson, *ibid.*, p. 1004.
8. G. G. Wanless, W. H. King, Jr., J. J. Ritter, *Biochem. J.* **59**, 684 (1955).
9. W. Carruthers and R. A. W. Johnstone, *Nature* **184**, 1131 (1959); Z. H. Kranz, J. A. Lamberton, K. E. Murray, A. H. Redcliffe, *Australian J. Chem.* **13**, 498 (1960); *ibid.* **14**, 264 (1961); R. Stevenson, *J. Org. Chem.* **26**, 5228 (1961); G. Eglinton, R. J. Hamilton, R. A. Raphael, A. G. Gonzalez, *Nature* **193**, 739 (1962).
10. R. O. Weenink, *Biochem. J.* **82**, 523 (1962); P. Mazliak, *Compt. Rend.* **252**, 1507 (1961).
11. E. Lederer, Sixth International Congress of Biochemistry, New York, 27 July 1964.
12. W. G. Meinschein, *Geochim. Cosmochim. Acta* **22**, 2 (1961). See also W. G. Meinschein, *Space Sci. Rev.* **2**, 653 (1963) and E. D. Evans, G. S. Kenny, W. G. Meinschein, E. E. Bray, *Anal. Chem.* **29**, 1858 (1957).
13. N. P. Stevens, E. E. Bray, E. D. Evans, in *Habitat of Oil*, L. G. Weeks, Ed. (Amer. Assoc. Petroleum Geologists, Tulsa, Okla., 1958), p. 779.
14. K. A. Kvenvolden, *Bull. Amer. Assoc. Petroleum Geologists* **46**, 1643 (1962).
15. E. E. Bray and E. D. Evans, *Geochim. Cosmochim. Acta* **22**, 2 (1961).
16. R. L. Martin, J. C. Winters, J. A. Williams, *Nature* **199**, 110 (1963).
17. L. S. Ciereszko, D. H. Attaway, M. A. Wolf, *Petroleum Research Fund, 8th Annual Report, 1963*, p. 33.
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Contact-Induced Cytotoxicity by Lymphoid Cells Containing Foreign Isoantigens

Abstract. *In tissue culture, immune lymph node cells containing foreign histocompatibility antigens of the H-2 type exert marked cytotoxic effects on tumor cells incompatible with the H-2 antigen. An equally pronounced effect is obtained when normal allogeneic and semi-isologous lymphoid cells of F_1 hybrids are caused to aggregate around the target tumor cells by treating the cultures with either heat-inactivated rabbit antiserum to mouse cells or phytohemagglutinin. Isologous lymph node cells have no effect. Thus, aggregation of lymphoid cells and target cells is a necessary but insufficient requirement for cytotoxicity in vitro; in addition, close contact must be established between histoincompatible cells.*

Immune lymphoid cells have a cytotoxic effect on various normal and neoplastic target cells grown in tissue culture (*1–4*). The effect was presumed to be due to the action of "cell-bound" antibodies, as opposed to humoral antibodies, since it did not require the participation of complement (*2–4*), which is necessary for the cytotoxic action of humoral antibodies. The possibility that the immune lymphoid cells secreted diffusable substances responsible for destroying the target cells was refuted by the demonstration that the cytotoxic effect was prevented by separating the two cell types with a cell-impermeable diffusion membrane (*5*). Also, there are marked differences between the kinetics of the cytotoxic action of immune lymphoid cells, which requires 24 to 48 hours, and the kinetics of the cytotoxic action of humoral antibodies, which requires as little as 1 hour. By the use of heavily x-irradiated target cells incapable of dividing, Wilson demonstrated that immune lymphoid cells actually killed the target cells and did not act by inhibiting their growth (*3*). Different authors have stressed the observation that close cellular contact between the immune lymphoid cells and the target cells always precedes demonstrable cytotoxicity. The importance of close contact is emphasized also by a recent report (*6*) demonstrating that the aggregation in vitro of normal allogeneic lymphoid cells and target kidney cells caused by phytohemagglutinin (PHA) resulted in the destruction of certain target cells. The experiments reported here have led to the conclusion that close contact between lymphoid cells and target tumor cells is a necessary but insufficient requirement for detectable cytotoxic effects in vitro; in addition it seems necessary for contact to be established between cells carrying different sets of H-2 (histocompatibility-2) antigens.

For this investigation tissue-culture systems were used in which cells from sarcomas induced by methylcholanthrene in mice of C57BL and (A × A.CA)F_1 hybrid origin were used as the targets. These cells are designated MC57S and MACD cells, respectively. The tumors were maintained by serial transplantation in isologous recipients. Cellular suspensions were obtained by treating finely minced tumor tissue with 0.25-percent solution of trypsin for 1 hour at room temperature in vitro. The cells were subsequently washed, and 10^6 cells in 1 ml of lactalbumin in Earle's medium supplemented with 10 percent calf serum (or, in later experiments, with the same amount of fetal calf serum) were added to each culture tube. The medium contained mykostatin (30 international units per milliliter) and penicillin (100 IU/ml). After 24 hours the medium was replaced with Parker-199 medium supplemented with 10 percent calf serum. In experiments designed for studying the possible cytotoxic effect of isoimmune and nonimmune lymphoid cells, the tubes were usually treated with 10×10^6 lymphoid cells immediately after the first change of the medium. The cultures were then incubated for 48 hours, after which they were treated with 1 ml of 0.25-percent solution of trypsin for 30 to 60 minutes at 37°C and subjected to repeated shaking. The mixtures were centrifuged, and part of the supernatant was removed. The tumor cells not stained by trypan blue were counted, and the volume of the supernatant was measured. Because of the pronounced differences

Table 1. Cytotoxic effects of normal and isoimmune lymph node cells from A.CA mice on sarcoma (target) cells in tissue culture in the presence of 10 percent heat-inactivated calf serum. The A.CA mice received one injection of normal cells from C57BL mice 6 to 10 days before the experiment (immunized), or they received no injection (control). (S.E., standard error.)

Expt. No.	Treatment of A.CA mice	No. of lymph node cells $\times 10^6$	No. of living tumor cells $\times 10^3 \pm$ S.E.	Living cells (%) and (cytotoxic index) in comparison with controls*
		MC57S cells as targets		
1	Immunized	10.0	34 ± 3.3	41.0 (0.59)
	Control	10.0	83 ± 11.9	100.0
2	Immunized	20.0	98 ± 25.1	30.7 (0.69)
	Immunized	10.0	110 ± 18.1	34.5 (0.66)
	Immunized	5.0	276 ± 46.5	96.5 (0.14)
	Control	20.0	319 ± 56.6	100.0
3	Immunized	20.0	162 ± 18.7	50.9 (0.49)
	Immunized	10.0	185 ± 17.4	58.2 (0.42)
	Immunized	5.0	193 ± 26.9	60.7 (0.39)
	Control	20.0	261 ± 11.0	82.1 (0.16)
	Control	10.0	266 ± 38.5	83.6 (0.16)
	Control	5.0	246 ± 22.8	77.9 (0.22)
			318 ± 15.9	100.0
		MACD cells as targets		
4	Immunized	10.0	35 ± 4.6	27.4 (0.73)
	Control	10.0	109 ± 11.5	85.1 (0.15)
			128 ± 22.4	100.0

* The cytotoxic index is defined as the difference between the percentages of living cells in the control group and in the experimental group divided by the percentage of living cells in the control group.

in size between the tumor cells and the lymphoid cells there was no difficulty in differentiating between them.

In several experiments, heterologous rabbit antiserum (to mouse cells) inactivated by heat was added to bring about aggregation of normal lymphoid cells and the target tumor cells. The antiserum was obtained from rabbits 7 days after they had received the last of four immunizing injections of living spleen and lymph node cells of (A × C3H)F_2 origin. The agglutinating titer against strain A red cells was 1/2000. The serums were always incubated for 30 minutes at 56°C before use and did not cause any cytotoxic effects on various nucleated target cells unless supplemented with complement.

The effect of immune lymphoid cells on allogeneic tumor cells was studied to determine the optimum conditions for cytotoxicity. The greatest number of cells were killed by immune lymph node cells, whereas spleen cells from the same donors were less efficient. The maximum effect was obtained 7 to 10 days after the last immunization. Inhibition of the cytotoxic effect occurred after treatment of the tumor cells with hyperimmune humoral antibodies prior to the addition of the immune lymph node cells, suggesting that production of humoral antibodies by the hyperimmune spleen cells was responsible for their limited effect (*7*).

These results led to the use of an experimental system consisting of lymph node cells from A.CA or A.SW mice immunized once with normal cells from C57BL mice (when MC57S sarcoma cells formed the target) or from strain A mice (in experiments with MACD cells) 6 to 10 days prior to the experiment; 5×10^6 to 20×10^6 lymph node cells per culture tube were added 24 hours after the tumor cells were explanted.

After treatment with immune lymph node cells, the percentage of target cells killed ranged from 96 percent to 26 percent in 20 different experiments, four of which are shown in Table 1. Increasing doses of immune lymph node cells usually increased the proportion of cells that were killed (Table 1). Although the immune lymphoid cells were thus highly active, normal lymph node cells regularly exerted a detectable, though weak, cytotoxic effect in relation to untreated target cells (Table 1), varying from 2 percent to 42 percent. Replacement of the calf serum with fetal calf serum markedly decreased the cytotoxic effect of the nonimmune allogeneic lymph node cells. When the two serums were separately agglutinated with red cells from strain A mice, a titer of 1/16 was obtained with the serum from the older calves, whereas no agglutination took place with the serum from fetal calves. In view of the importance ascribed to the contact between the lymphoid cells and the target tissue, it seemed possible that the heterologous antibodies present in the calf serum caused a mixed agglutination of the nonimmune lymph node cells and the target sarcoma cells, resulting in death of the target cells. Direct experiments were performed to test this possibility.

Rabbit serum strongly reactive against mouse cells was mixed with cultures of the tumor cells to give total dilutions of 1/10 to 1/20, 1 hour before the addition of normal allogeneic lymph node cells or, in control cultures, the addition of isologous cells of the same type. Other controls were prepared simultaneously as shown in Table 2. The treatment of the target tumor cells with heterologous antiserum had no significant effect on the number of living cells after 48 hours, whereas the addition of allogeneic lymph node cells to the cultures treated with heterologus antiserum resulted in a marked cytotoxic effect on the tumor cells (Table 2). Nonimmune allogeneic lymph node cells had no effect in the absence of heterologous immune serum. Similarly, isologous lymph node cells did not induce detectable cytotoxic effects when tested in the presence of heterologous antiserum. Morphological studies revealed a pronounced aggregation of allogeneic and isologous lymph node cells as well around the target cells. Since the isologous cells were not active cytotoxically, it appears that aggregation as such is not sufficient for cytotoxicity.

That the detectable cytotoxic effects occurred only after heterologous antibody-induced contact between histoincompatible lymph node cells and target cells can be accounted for by various hypotheses. Thus, it might be assumed that the heterologous antiserum stimulated the allogeneic lymph node cells to perform a primary immune response in vitro, in analogy with the assumed capacity of PHA to initiate an immune response in vitro (*8*). To investigate this possibility, semi-isologous F_1 hybrid cells of the genotype (A.CA × C57BL)F_1, genetically incompetent to react immunologically against the target tumor cells of C57BL origin, were admixed to the cultures of MC57S sarcoma cells treated with heterologous antibody (Table 2, experiments 3 and 4). As before, the isologous lymph node cells were incapable of inducing cytotoxicity, whereas both the allogeneic and the semi-isologous F_1 hybrid cells caused a pronounced cytotoxic effect. A similar experiment was performed with PHA instead of heterologous antiserum (Table 2, experiment 5); aggregation of lymphoid cells and target tumor cells occurred in a manner

similar to that caused by rabbit antiserum. Thus, the first possibility appears unlikely. It is more probable that the cytotoxic effect was caused by the contact between target tumor cells and lymph node cells containing foreign histocompatibility antigens of either the H-2 type or some other type.

Several independent findings indicate that marked differences exist between the mechanisms of target cell destruction caused by humoral antibodies and of that caused by immune lymphoid cells, suggesting that the two immunological reactions are based on different biological phenomena. The repeated demonstrations of aggregation of the immune lymphoid cells around the target cells prior to their destruction suggests that close contact between the two cell types is a prerequisite for cytotoxicity. This suggestion is strengthened by the recent findings (*7*) that prior treatment of the target cells with serum antibodies, capable of reacting with the antigenic determinants of the tumor target and therefore of blocking the site of attachment of the immune lymphoid cells, partly or completely inhibits the cytotoxic effect. Furthermore, the finding that aggregation of normal allogeneic lymphoid cells and target kidney cells induced by PHA causes destruction of the target cells (*6*) emphasizes the necessity for close cellular interaction in cytotoxicity. The present findings indicate, however, that close cellular contact in the form of mixed agglutinates is not sufficient by itself to cause the destruction of target cells. Pronounced cytotoxic effects on the target cells occurred only in aggregates made up of lymph node cells containing foreign histocompatibility antigens, even in situations where the lymph node cells could not carry out immunological reactions because the target cells did not contain any foreign histocompatibility antigens. It has been demonstrated (*7*) that normal parental lymphoid cells can kill semi-isologous F_1 hybrid target cells in the presence of PHA. In this case the target cells do not meet any foreign antigens. It seems possible, however, that structural discrepancies exist between the two cells and the cytotoxicity would depend, therefore, on structural rather than antigenic incompatibility.

The present findings have certain resemblances to the syngeneic preference phenomenon described by Hellström and found with tumor cells in vivo and in vitro (*9, 10*). The growth of parental tumor cells was inhibited by exposure to foreign H-2 antigens in a semi-isologous hybrid recipient incapable of reacting immunologically against the tumor, in comparison with the same tumor cells in homozygous parental recipients (*9*). The phenomenon could be reproduced in vitro by treating tumor cells with extracts of foreign antigens which inhibited growth of the cells (*10*). In the present experiments, the absence of cytotoxic effects when tumor cells were treated with allogeneic lymph node cells without the addition of heterologous antiserum might be due to the failure of two incompatible living cells to establish close contact without the aid of agents such as heterologous antiserum and phytohemagglutinin.

These results suggest that the cytotoxic effect of immune lymphoid cells on various target cells in vitro is caused by close contact between two incompatible cells, leading to the death of the target cells, and probably also of the immune lymphoid cells. The cause of cell death would be attributable to the cells being antigenically or structurally incompatible, or both. The part played by immunological reactions in cytotoxicity in vitro would be limited, therefore, to specific receptors being present on the immune lymphoid cells, making possible attachment to the target cells. Whether this explanation would also be valid for the effect of immune lymphoid cells in vivo is not known.

Table 2. Cytotoxic effect of normal allogeneic and semi-isologous F_1 hybrid lymph node cells on sarcoma cells (MC57S cells in each experiment) in tissue culture subsequent to mixed agglutination as a result of treatment with heterologous antiserum or phytohemagglutinin (PHA). The antiserum, obtained from rabbits immunized with cells from (A × C3H)F_2 mice, was diluted from 1/10 to 1/20. Serum from nonimmunized (normal) rabbits was also diluted 1/10 to 1/20.

Expt. No.	Origin of normal lymph node cells	Type of serum or PHA	No. of living tumor cells × 10^3 ± S.E.	Living cells (%) and (cytotoxic index) in comparison with controls
1		Antiserum	202 ± 13.8	73.2 (0.27)
	A.CA	Antiserum	93 ± 11.2	33.6 (0.66)
	A.CA		228 ± 13.9	82.8 (0.17)
			276 ± 30.4	100
2			113 ± 15.2	97.5 (0.03)
		Normal serum	116 ± 14.4	100
	A.CA		109 ± 19.1	94.0 (0.06)
	C57BL		143 ± 20.5	123.3 (negative)
	A.CA	Normal serum	108 ± 10.4	93.1 (0.07)
	C57BL	Normal serum	167 ± 16.0	144.0 (negative)
	A.CA	Antiserum	43 ± 7.4	37.1 (0.63)
	C57BL	Antiserum	129 ± 23.8	111.2 (negative)
3		Antiserum	112 ± 21.5	81.8 (0.18)
		Normal serum	137 ± 15.3	100
	A.CA	Normal serum	134 ± 13.3	97.8 (0.02)
	(A.CA × C57BL)F_1	Normal serum	114 ± 13.0	83.2 (0.17)
	C57BL	Normal serum	111 ± 17.7	81.0 (0.19)
	A.CA	Antiserum	61 ± 11.7	44.5 (0.56)
	(A.CA × C57BL)F_1	Antiserum	69 ± 10.5	50.4 (0.50)
	C57BL	Antiserum	105 ± 5.8	76.6 (0.23)
4		Antiserum	38 ± 3.9	100
	A.CA	Antiserum	15 ± 2.2	39.7 (0.60)
	(A.CA × C57BL)F_1	Antiserum	13 ± 1.2	32.9 (0.67)
	C57BL	Antiserum	41 ± 9.8	107.1 (negative)
5		PHA*	110 ± 20.5	115.8 (negative)
	A	PHA	32 ± 6.9	33.7 (0.66)
	(A × C57BL)F_1	PHA	58 ± 7.4	61.1 (0.40)
	C57BL	PHA	84 ± 6.5	88.4 (0.12)
			95 ± 17.3	100
	A		74 ± 10.7	77.9 (0.22)
	(A × C57BL)F_1		105 ± 10.5	110.5 (negative)
	C57BL		106 ± 8.2	111.6 (negative)

* The phytohemagglutinin (Batch K4912, Wellcome Research Laboratories, Beckenham, England) was used in a total dilution of 1/50.

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References and Notes

1. A. Govaerts, *J. Immunol.* **85**, 16 (1960); H. Koprowski and M. W. Fernandes, *J. Exptl. Med.* **116**, 467 (1962); P. Perlmann and O. Broberger, *ibid.* **117**, 717 (1963).
2. W. Rosenau and H. D. Moon, *J. Natl. Cancer Inst.* **27**, 471 (1961).
3. D. B. Wilson, *J. Cell. Comp. Physiol.* **62**, 273 (1963).
4. B. D. Brondz, *Folia Biol. Prague* **10**, 164 (1964).
5. W. Rosenau, in *Cell-Bound Antibodies*, B. Amos and H. Koprowski, Eds. (Wistar Institute, Philadelphia, 1963), p. 75.
6. G. Holm, P. Perlmann, B. Werner, *Nature* **2031**, 841 (1964).
7. E. Möller, in preparation.
8. K. Hirschhorn, F. Bach, R. L. Kolodny, *Science* **142**, 1185 (1963).
9. K. E. Hellström, *Nature* **199**, 614 (1963).
10. ———, I. Hellström, G. Haughton, *ibid.* **209**, 661 (1964).
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