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Mapping of Sites on the Surface Membrane of Mammalian Cells

II. Relationship of Sites for Concanavalin A and an Ornithine, Leucine Copolymer*

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Summary. Cells transformed by Simian Virus 40 have sites on the surface membrane for Concanavalin A (Con A) and a copolymer of ornithine, leucine (POL). The cells can be rapidly agglutinated by Con A, more slowly aggregated by POL, and they can be killed by both compounds. Treatment with Con A or POL has been used to select resistant cell variants from the transformed cells. Variants selected for resistance to Con A were also resistant to POL, but variants selected for resistance to POL were not resistant to Con A. The POL-selected variants showed less aggregation by POL but no decrease in agglutinability by Con A, whereas Con A-selected variants showed a decrease both in POL aggregation and Con A agglutination. The selection for both sites by Con A and only for POL sites by POL, can be explained in that the sites for POL are part of the sites for Con A and/or are included in clusters of Con A sites.

There are sites on the surface membrane of cells transformed by Simian Virus 40 (SV 40) that can interact with Concanavalin A (Con A) and a copolymer of orthine, leucine (POL). After interaction with these sites, the cells can be rapidly agglutinated by Con A (Inbar & Sachs, 1969*a*, *b*; Inbar, Ben-Bassat & Sachs, 1971*b*, 1972*a*; Ben-Bassat, Inbar & Sachs, 1971) and more slowly aggregated by POL (Duksin, Katchalski & Sachs, 1970). Both compounds can also produce cell toxicity (Shoham, Inbar & Sachs, 1970; Inbar, Ben-Bassat & Sachs, 1971*a*, 1972*b*; Duksin *et al.*, 1970). The present experiments were undertaken to map the relationship between sites for Con A and POL. The method used was to select from SV 40 transformed cells, resistant variants by treatment with either Con A or POL. The variants were then tested to determine (1) whether selection for resistance to one compound was associated with a gain of resistance to the other

* Paper I in this series is Inbar, Ben-Bassat and Sachs (1971*a*).

compound, and (2) to show to what extent selection for resistance was associated with a decrease in agglutinability by Con A and aggregation by POL.

Materials and Methods

Cell Cultures

The cell line used in the present experiments was a clone derived from an SV 40-induced hamster tumor. The cells were grown in Eagle's medium with a fourfold concentration of amino acids and vitamins (EM) with 10% fetal calf serum in 50-mm plastic petri dishes (Nunc). The cells were routinely subcultured in 0.25% trypsin solution (Difco 1:300) every 3 to 5 days. There was no detectable mycoplasma contamination as shown by testing the cultures on mycoplasma agar according to Chanock, Hayflick and Barile (1962).

Concanavalin A (Con A) and the Copolymer of Orthine, Leucine (POL)

Con A was prepared from Jack bean meal by two crystalizations (Sumner & Howell, 1936). The POL used (supplied by D. Duksin) was prepared as described (Duksin *et al.*, 1970) and was a random copolymer of L-orthine and L-leucine (molar ratio 1:1) with an average molecular weight of 5,000 to 7,000 as measured by sedimentation in dimethylformamide.

Selection of Variants Resistant to Con A

Transformed cells were seeded in EM with 10% serum, and after 24 hr, when the cell number was about 0.5×10^6 per 50-mm petri dish, the cultures were washed twice with phosphate-buffered saline (PBS), pH 7.2. A sample of 1.5 ml of 400 μg/ml Con A diluted in EM (without serum) was added and the cultures were incubated for 7 hr at 37 °C. After this 7-hr period, 2.5 ml of EM with serum was added to give a final serum concentration of 10% serum and the cultures were further incubated at 37 °C. Twenty-four hours after addition of Con A, the medium was removed and the cells were incubated for 25 min at 37 °C in 2 ml 0.1 M α-methylglucoside. This was then replaced by fresh medium with 10% serum. The cells were incubated to form colonies, and colonies with a more epitheloid type of cell than the parental transformed cells were isolated. The same procedure was used for two more successive selections. Colonies isolated after three selections were subcultured 2 to 3 times, before testing the properties of the cells.

Selection of Variants Resistant to POL

Twenty-four hours after seeding the transformed cells in EM with 10% fetal calf serum, when there were about 0.5×10^6 per 50-mm plate, the cultures were washed twice with PBS, and 250 μg/ml POL in 1.5 ml EM (without serum) was added. After 2-hr incubation at 37 °C, the cultures were washed 3 times with PBS and fresh medium with 10% serum was added. The cells were incubated to form colonies, and colonies with a more epitheloid type of cell than the parental transformed cells were isolated. The same procedure was used for two more successive selections. Colonies isolated after the third selection were subcultured 2 to 3 times before the cells were tested for their properties.

Assays for Agglutination by Con A and Aggregation by POL

For both assays, 0.5×10^6 cells were seeded per 90-mm petri dish in EM with 10% serum. To test for agglutination by Con A, the cells were washed twice with PBS and removed from the petri dish with a solution of 0.02% disodium versenate (Inbar & Sachs, 1969*a*). The suspended cells were washed twice with PBS, and then diluted in PBS at a concentration of 1 to 1.5×10^6 cells per ml. A sample of 0.5 ml of the Con A solution was mixed with 0.5 ml of the cell suspension in a 35-mm petri dish, and the density and size of aggregates was scored in a scale of $-$ to $+ + + +$ after 30-min incubation at room temperature. Trypsin-treated cells were washed twice with PBS, removed from the plate by incubating for 20 min at 37 °C with a 0.25% trypsin (1:300) solution, washed with PBS and tested for agglutination.

To test for aggregation by POL, cells were suspended in EM with 10% serum at a concentration of 1×10^6 cells per ml, POL was added, and 0.1 ml of the cell suspension was introduced into a small well (13-mm diameter) in a glass slide and the well was covered with a cover slip. The appearance of aggregates was scored 24 hr after incubation at 37 °C (Duksin *et al.*, 1970).

Results

Increased Resistance in Successive Cycles of Selection with Con A or POL

Of the 0.5×10^6 transformed cells treated with 400 μg/ml Con A, an average of 40 cells formed colonies in the first selection. Eight colonies were isolated, subcultured twice and each colony was again treated with 400 μg/ml Con A. Eight of the surviving colonies, one from each original colony, were again treated with 400 μg/ml Con A for the third cycle of selection. In contrast to an average of 40 surviving colonies after treatment in the first selection cycle, there were 62 surviving colonies after the second cycle and 95 after the third selection. Three colonies from the third selection were used for further studies.

In the selection with POL, an average of 10 colonies survived the first treatment with 250 μg/ml POL. Two colonies were isolated and subcultured, and each colony was again treated with 250 μg/ml POL. Two colonies, one from each original colony, were treated under the same conditions for the third selection. In the second selection, an average of 50 colonies survived from the first and 35 from the second colony. In the third cycle of selection, 83 and 52 colonies survived, respectively. Two colonies from the third selection were isolated, subcultured, and used for further studies.

Resistance to Con A

Resistance to Con A was measured by adding 35, 70 or 100 μg/ml Con A in 1.5 ml EM (without serum) to cells 24 hr after seeding. After 7 hr at 37 °C, 2.5 EM with fetal calf serum to give a final concentration of 10% serum was

Table 1. Resistance of Con A and POL variants to Con A

Cells	Concentration of Con A (μg/ml)	No. of cells per plate $\times 10^{-6}$ at		
		24 hr	48 hr	72 hr
Transformed	0	1.4	3.2	6.5
	35	1.1	0.9	0.7
	70	0.8	0.7	0.5
	100	0.7	0.6	0.5
Variant 1	0	1.3	1.9	2.2
(selected with Con A)	35	1.0	1.2	1.6
	70	0.8	1.2	1.5
	100	0.7	0.9	1.4
Variant 4	0	1.2	1.8	2.3
(selected with POL)	35	0.6	0.8	0.7
	70	0.6	0.6	0.7
	100	0.4	0.4	0.4

Con A was added 24 hr after seeding, when the cell number was 0.6×10^6 per 50-mm petri dish, and the cells were counted every day.

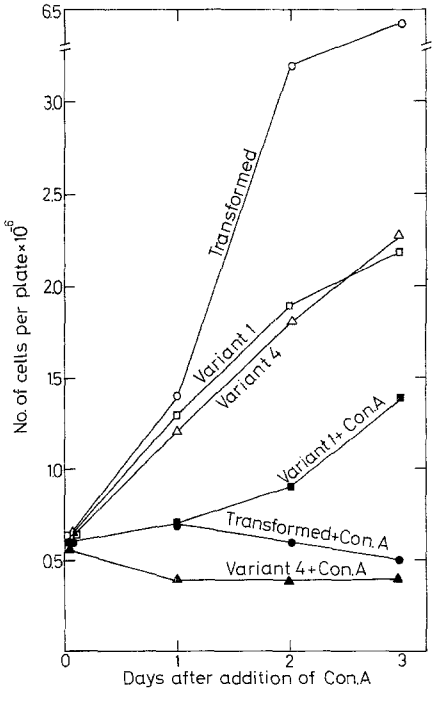

Fig. 1. Growth of variants 1, 4 and transformed cells after treatment with 100 μg/ml Con A. Open symbols = untreated, closed symbols = treated with Con A

added, and the cells were incubated for 3 days. The number of cells was counted each day. Cells from the three Con A variants (variants 1, 2 and 3) were able to multiply during the 3 days, although at a lower rate than the untreated cells, at all the tested concentrations of Con A. But there was no such multiplication after Con A treatment of either the POL variants (variants 4 and 5) or the transformed cells. Similar results were obtained with each of the 3 Con A and the 2 POL variants, and the data for one variant from each group (variants 1 and 4) are shown in Table 1 and Fig. 1.

Resistance to POL

Resistance to POL was measured by adding 25, 50 or 80 μg/ml POL to cells 24 hr after seeding in EM with 10% serum. The cells were counted each day for 3 days. With 80 μg, the transformed cells showed an initial high per cent of cell killing and no subsequent increase in the total number of cells during the 3 days. However, the Con A and POL variants showed a lower initial per cent of cell killing, and the cells were then able to multiply. There was the same initial killing and subsequent growth with both types of variants. After treatment with 25 or 50 μg/ml POL, there appeared to be an initial killing with the Con A but not with the POL variants.

Each of the three Con A and the two POL variants gave similar results, and the data for one variant from each group (variants 1 and 4) are shown in Table 2 and Fig. 2.

Agglutinability by Con A

Agglutination by Con A was tested at 1 to 4 days after seeding. With the culture conditions used, even transformed cells are not agglutinated by Con A at 1 day after seeding (Ben-Bassat *et al.*, 1971; Inbar *et al.*, 1972*a*), and this was also found in the present experiments. On the second day, the transformed cells and POL variants were agglutinated at all Con A concentrations tested, whereas the Con A variants were less agglutinable (Fig. 3). After treatment with trypsin, the Con A variants were as agglutinable as the transformed cells and POL variants. On the third and fourth day, the variant cells were still in clumps after the cells were removed from the petri dish with disodium versenate, so that agglutination by Con A could not be tested.

Aggregation by POL

Aggregation with 25 μg/ml POL was determined at 1 and 3 days after seeding. Although the transformed cells were aggregated, both the Con A

Table 2. Resistance of Con A and POL variants to POL

Cells	Concentration of POL (μg/ml)	No. of cells per plate $\times 10^{-6}$ at		
		24 hr	48 hr	72 hr
Transformed	0	1.2	2.8	6.3
	25	0.4	1.0	1.8
	50	0.2	0.4	1.0
	80	0.05	0.05	0.05
Variant 1	0	1.1	1.8	2.1
(selected with Con A)	25	0.5	0.8	1.5
	50	0.3	0.7	1.1
	80	0.3	0.6	1.0
Variant 4	0	1.1	1.9	2.2
(selected with POL)	25	0.8	1.4	1.9
	50	0.7	1.2	1.6
	80	0.3	0.6	1.1

POL was added 24 hr after seeding, when the cell number was 0.6×10^6 per 50-mm petri dish, and the cells were counted every day.

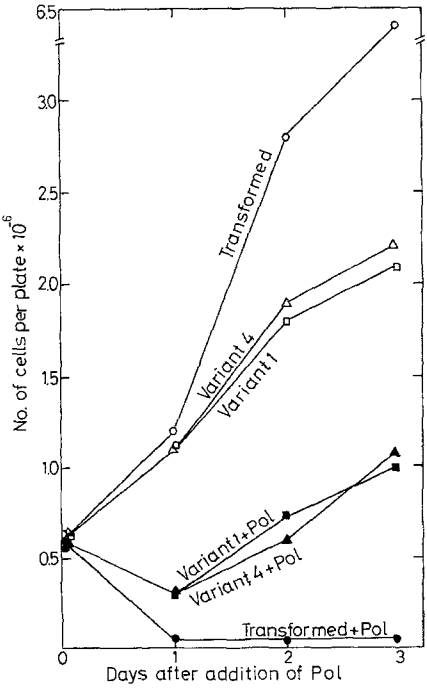

Fig. 2. Growth of variants 1, 4 and transformed cells after treatment with 80 μg/ml POL. Open symbols = untreated, closed symbols = treated with POL

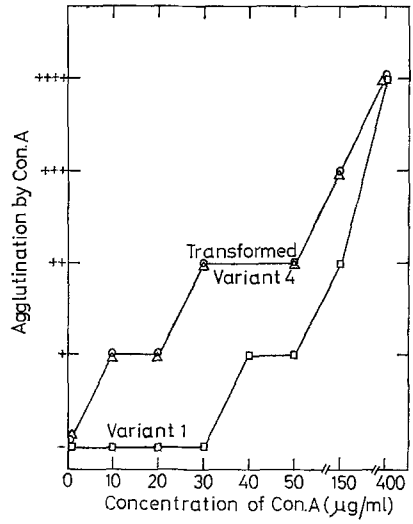

Fig. 3. Agglutinability by Con A of variants 1, 4 and transformed cells at 2 days after seeding

and POL variants showed no aggregation at this concentration of POL. Similar results were obtained at 1 and 3 days.

Growth Properties of Variants

The Con A and POL variants both had a more epitheloid morphology than the transformed cells, but the variants showed the same initial growth rate as the transformed cells in nonconfluent cultures. Both types of variants had, compared to the transformed cells, a lower saturation density, cloning efficiency in fluid medium, soft agar, and at 41 °C, a decreased tumor formation in adult animals, and a larger cell size (Table 3). All the Con A and POL variants showed, like the transformed cells, the presence of SV 40 specific T-antigen. The growth properties of the variants, and their degree of resistance to Con A and POL, was maintained during 10 subcultures of the cells.

Discussion

To map the relationship of sites for Con A and POL on the cell surface membrane, treatment with Con A or POL was used to select resistant cell variants from SV 40-transformed cells, and these variants were then tested for cross resistance. The results have shown that the variants selected for resistance to Con A were also resistant to POL, but that variants selected for resistance to POL were not resistant to Con A. The Con A-selected

Table 3. Growth properties of Con A and POL variants

Cells	Selected with:	Plating efficiency (%)[a]	Saturation density $\times 10^{-6}$	Cloning efficiency (%)			Cell size	Tumor formation in adult animals			
				In fluid medium at 37 °C	In soft[b] agar at 37 °C	Colonies[c] at 41 °C	μ^3[d] $\times 10^{-3}$	No. of cells inoculated per animal			
								10^1	10^2	10^3	10^4
Transformed	—	95	6.5	78	25	35	5.4	1/5[e]	3/5	5/5	5/5
Variant 1	Con A	70	1.8	25	3	2.3	15.2	0/5	0/5	1/5	3/5
Variant 2	Con A	72	2.0	30	6	3.5	14.0	0/5	0/5	2/5	3/5
Variant 3	Con A	78	2.1	53	7	0	15.6	0/5	0/5	3/5	4/5
Variant 4	POL	69	2.1	28	10	5.8	14.2	0/5	0/5	3/5	4/5
Variant 5	POL	71	2.3	30	8	0	15.6	0/5	0/5	2/5	4/5

[a] Percentage of cells attached to the tissue culture petri dish at 6 hr after seeding.

[b] (Macpherson & Montagnier, 1964).

[c] $\frac{\text{No. of colonies at 41 °C}}{\text{No. of colonies at 37 °C}} \times 100$ (Rabinowitz & Sachs, 1970).

[d] Cell volume was measured by centrifuging a known number of cells in a centrifuge tube containing a graduated capillary tube of 1-mm diameter. After 20-min centrifugation at $300 \times g$, the cells had pelleted in the capillary tube and the volume was calculated assuming that cells are spherical (Inbar & Sachs, 1969*b*).

[e] No. of animals with tumors/No. of animals inoculated. Three days after seeding, the cells were inoculated subcutaneously in 0.2 ml PBS into adult hamsters, and the animals observed for progressively growing tumors for 50 days.

variants were less agglutinable by Con A and showed less aggregation by POL than the transformed cells, whereas POL-selected variants only showed less aggregation by POL. The results indicate that there is a relationship between the sites for Con A and POL on the surface membrane, so that selection with Con A selects for both sites and selection with POL selects only for POL sites. This suggests that the sites for POL are part of the sites for Con A and/or that they are included in the Con A sites that may occur in clusters (Ben-Bassat *et al.*, 1971; Nicolson, 1971). The relationship of Con A sites and transport sites for amino acids and carbohydrates, has previously been described (Inbar *et al.*, 1971*a*).

The POL- and Con A-selected variants tested, like other Con A-selected variants (Ozanne & Sambrook, 1971) and variants induced by another procedure (Rabinowitz & Sachs, 1968, 1970; Hitotsumachi, Rabinowitz & Sachs, 1971) showed a reversion of properties characteristic of transformation without loss of the virus genome. It will be of interest to use selection for resistance to other compounds to map the relationship of other sites on the surface membrane, and to determine whether resistance is always associated with a reversion of transformed properties.

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