Hydrogen abstraction

$$R_2CHCCl_3 \xrightarrow{\text{light}} R_2CH\dot{C}Cl_2 + \dot{C}l \quad (1)$$

$$R_2CH\dot{C}Cl_2 + CH_3OH \longrightarrow R_2CHCHCl_2 + \dot{C}H_2OH \quad (2)$$

$$R_2CHCHCl_2 \xrightarrow{\text{light}} R_2CH\dot{C}HCl + \dot{C}l \quad (3)$$

$$R_2CH\dot{C}HCl + CH_3OH \longrightarrow R_2CHCH_2Cl + \dot{C}H_2OH \quad (4)$$

In the presence of oxygen

$$R_2CH\dot{C}Cl_2 + O_2 \longrightarrow R_2CHCCl_2(\dot{O}_2) \quad (5)$$

$$2R_2CHCCl_2(\dot{O}_2) \longrightarrow 2R_2CHCCl_2\dot{O} + \dot{O}_2 \quad (6)$$

$$R_2CHCCl_2\dot{O} \longrightarrow R_2CHCOCl + \dot{C}l \quad (7)$$

$$R_2CHCOCl + CH_3OH \longrightarrow R_2CHCOOCH_3 + HCl \quad (8)$$

$$R_2CHCCl_3 + \dot{C}l \longrightarrow R_2\dot{C}CCl_3 + HCl \quad (9)$$

$$R_2\dot{C}CCl_3 + O_2 \longrightarrow R_2C\dot{O}_2CCl_3 \quad (10)$$

$$2R_2C\dot{O}_2CCl_3 \longrightarrow 2R_2C\dot{O}CCl_3 + O_2 \quad (11)$$

$$R_2C\dot{O}CCl_3 \longrightarrow R_2CO + \dot{C}Cl_3 \quad (12)$$

($R = p$-$Cl \cdot C_6H_4$)

2 (M + 2), and so forth, as ^{35}Cl and ^{37}Cl occur in the ratio of 3 : 1. Many of the oxidation products are known; therefore, mass spectral and chromatographic comparisons could be made with authentic samples. Mass spectral studies were extended to model compounds, and the photochemistry of *p,p'*-dichlorobenzophenone was also investigated to obtain information on the further breakdown of photolytic products. Substitution of an oxygen atom at C-1 of the 1,1-diphenylethane system affords aboundant fragments at *m/e* 139 ($^{35}ClC_6H_4CO$) and *m/e* 111 ($^{35}ClC_6H_4$).

Molecular ions were obtained in the majority of cases, but difficulty was encountered with (1). An intense base peak at *m/e* 265 suggested the oxonium ion structure (1A) presumably formed by loss of a methoxycarbonyl residue from (1). The presence of this residue in the isolated material was confirmed by the infrared and nuclear magnetic resonance spectra of the isolated fraction.

The compound with *m/e* 338, which was present in significant quantity in the photooxidation products of DDE, could not be assigned a complete structure on the available evidence. However, nuclear magnetic resonance, infrared, and mass spectral data suggest that it contains a methyl dichlorofluorenyl carboxylate moiety.

Mass spectral data alone were used for assignment of structure where trace quantities were obtained and authentic material was unavailable.

Most of the photolysis products could be examined with these techniques. Compounds of higher molecular weight, such as those described by Fleck (*1*), are probably present in small quantity. The seqence of reactions of free radicals postulated by Mosier *et al.* (1) explains the photolytic pathway. There are many instances of photolytic fission of a C–Cl bond to form a radical which can subsequently react with solvent by hydrogen abstraction (*6*). We postulate that, in the presence of excess oxygen, photochemically generated free radicals from DDE and DDT add oxygen. Rearrangement and reaction of unstable intermediates account for the formation of the photooxidation products which we have identified.

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Duplicate Plating of Immune Cell Products: Analysis of Globulin Class Secretion by Single Cells

Abstract. *Antibodies secreted by individual immune cells were collected focally in very thin "original" and "imprint" layers of agar containing the target antigen, sheep erythrocytes. Identical treatment of both layers led to mirror image patterns of hemolytic plaques. Development of one layer for immunoglobulin M hemolysins and the other for immunoglobulin G hemolysins produced unrelated plaque patterns indicating that few, if any, cells simultaneously release substantial amounts of both γM and γG antibodies.*

A comprehensive understanding of the synthetic capacities of individual immune cells depends upon the facility with which the products of single cells may be collected and analyzed. Single cell isolation procedures permit such sampling, but the numbers of cells that can be examined are relatively few (*1*). Fluorescent antibody techniques also permit reasonable inferences about immunoglobulin synthesis since they detect residual globulins in or on the cells of fixed tissue specimens (*2*). Studies of single cells by the isolation procedure showed that up to 19 percent of immune cells may produce two classes of immunoglobulins simultaneously (*1*), while with fluorescent antibody techniques instances of possible double producer cells have ordinarily averaged around 1 percent (*2*). We now report a new replica-plating procedure which allows duplicate sampling of the products of single viable immune cells without necessitating isolation of single cells.

Adult female New Zealand White rabbits were immunized intravenously with 10-ml portions of thrice-washed 10 percent sheep erythrocytes on days 0, 9, 18, and 24. Suspensions of immune cells (*3*) were obtained by processing the whole spleens of these animals on days 5, 6, 7, 8, and 27. The cells were washed by centrifugation, and those from two rabbit spleens were pooled and suspended in 20 ml of cold Hanks solution. Very thin layers of sheep erythrocytes in agar were prepared as follows. A mixture (at 45°C) composed of 18 ml of 1.1 percent liquified Bactoagar (Difco) in isotonic 0.05*M* tris-buffered (*p*H 7.4) Eagle's minimum essential culture medium

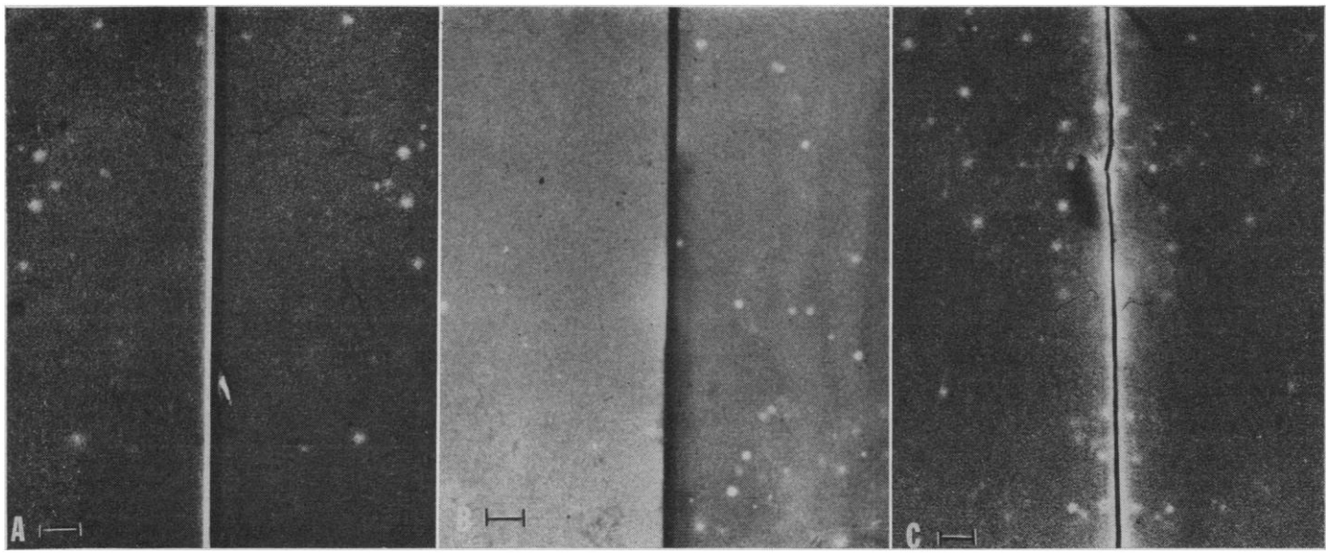

Fig. 1. Comparison of plaque patterns in original and imprint layers developed for plaques due to cells releasing antibody of high or of low hemolytic efficiency. (A) Both the original and imprint layers were developed directly (D-D) for plaques of high hemolytic efficiency yielding mirrored plaque patterns. (C) Both layers were developed indirectly (I-I) for plaques due to cells releasing antibody of low hemolytic efficiency yielding similar mirrored plaque patterns. (B) Development of one layer for plaques of high and the other for plaques of low hemolytic efficiency (D-I) led to unrelated plaque patterns. Markers in all cases indicate 1 mm.

(MEM); 1.5 ml of 1 percent (weight to volume) diethylaminoethyl-dextran (Pharmacia, Uppsala, Sweden) in 0.15*M* NaCl; and 0.8 ml of thrice-washed sheep erythrocytes (SRBC), 50 percent in 0.15*M* NaCl was placed onto a previously prepared solid base-layer of 2.2 percent Bactoagar in MEM in a shallow leucite tray (37.5 by 12 by 4 cm). The tray was briskly rocked and tilted for up to 20 seconds or until the thin (approximately 0.1 mm) SRBC-liquid-agar layer coated the entire base-agar layer. The trays were cooled on a level table at room temperature for 10 minutes, slabs (7 by 7.5 cm) were cut from the center of the solid agar preparations, and four 0.05-ml droplets of an immune cell suspension were delivered onto the SRBC surface of half of them. Immediately thereafter the remaining slabs—"imprint layers"—were superimposed on the "originals." Gentle external stroking was used to spread the immune cells between the two apposed agar-SRBC layers and to express trapped air bubbles at the edges. The slabs, supported on glass lantern slides (8 by 10 cm), were incubated at 37°C for 2 hours in humid chambers and then overnight at 3°C. The still superimposed slabs were then cut into five strips (1.5 by 7 cm). The original and imprint layers of each strip were then separated, and their contained antibody foci developed as plaques of localized hemolysis either directly by a 30-minute exposure at 37°C to 10 percent guinea pig complement or indirectly by a 30-minute exposure to a 1:60 dilution in 0.15*M* NaCl of a specially selected sheep antiserum to rabbit γ-globulin (*4*) followed by a 0.15*M* NaCl rinse and an identical exposure to 10 percent guinea pig complement. Developed original and imprint strips were then aligned side by side and photographed; the plaque patterns of respective photographic prints were evaluated by marking in red the size and position of each "original" layer plaque on a transparent celluloid sheet, inverting the sheet, and marking in black the size and position of each imprint layer plaque. Juxtaposed red and black marks identified those plaques represented bilaterally. Such bilateral representation of individual plaques was considered satisfactory evidence of duplicate sampling of the immunoglobulin products from single cells. Plaques developed only unilaterally were represented by spatially discrete black or red marks. In order to assess the efficiency of duplicate sampling from single cells, the original and imprint layers of both the extreme right- and extreme left-hand strips from each slab preparation were developed directly for the appearance of plaques due to cells producing high hemolytic efficiency (γM) antibody (*5*, *6*) while both the "original" and "imprint" layers of the middle strip from each slab were developed indirectly for detection of plaques caused by cells releasing antibody of low hemolytic efficiency (γG) antibody (*5*). When enough mirror-image plaques developed in the control assay strips (45 to 80 percent) for a given slab, the definitive analyses were done on the photographs of the intervening second and fourth strips from the same slab; for each of these, one layer had been developed directly for γM plaques and its apposed layer indirectly for γG plaques. In these instances the bilateral appearance of any given plaque would be evidence for a cell simultaneously secreting both γM and γG antibodies. This is so because γG plaques do not appear on direct assay layers and because the facilitating sheep antiserum to rabbit globulin was unique in its capacity to fully inhibit the appearance of γM plaques in the indirect assay layers (*4*).

Results of representative assay pairs are pictured in Fig. 1. When both original and imprint layers were developed directly (D-D) for γM detection, the majority of plaques were represented bilaterally yielding a mirror-image plaque pattern. The same predominantly mirror-image type pattern was routinely manifest when both layers of a strip were developed indirectly (I-I) for detection of plaques due to cells releasing γG antibodies. In intervening strips with one layer developed directly and its apposed layer developed indirectly (D-I) the mirrored configuration of plaque patterns no longer obtained. Bilateral spatial correspondence of individual plaques was an extremely rare event in D-I strips, as can be seen from Table 1 which records the observations on seven separate pools of im-

mune cells derived from rabbits assayed on days 5 to 8 after the first immunization and from other rabbits assayed after four immunizations.

The efficiency of bilateral sampling of the products of single immune cells can be judged by the precentage of bilateral plaque correspondence in the D-D and I-I assays. In these assay pairs, plaque correspondence was 47 to 83 and 46 to 69 percent, respectively. In each case, plaque correspondence averaged about 60 percent. Contrastingly, the bilateral plaque correspondence in D-I assay pairs was 0 to 1.77 percent and averaged only 1.16 percent, indicating that γM and γG plaque production derives largely, if not exclusively, from two separate immune cell populations. (The occasional coincidence of a γM-producing cell with a γG-producing cell must also contribute to this average.) The number of γM plaques far exceeded the number of γG plaques in D-I assays on day 5 after the first immunization, but by days 6 and 7 the ratio of their numbers commonly approached unity—an indication that the transition from γM to γG antibody production was well under way. By day 8, γG plaques outnumbered γM plaques. If numerous individual cells convert from γM to γG production during this period, such an event must for most cells take place quite abruptly since the change in bilateral plaque correspondence is inappreciable. This indicates that few, if any, rabbit spleen cells can simultaneously release comparable quantities of both γM and γG hemolysins. Such an argument appears more cogent in view of the observations on hyperimmune spleen cells, which indicate that the percentage (and range) of bilateral plaque correspondence in D-I assays is essentially unchanged even at a time well past maximum transition, when γG-producing cells predominate.

In an idealized replica-imprint assay, two perfectly smooth and flat indicator layers of SRBC are separated only by a monolayer of viable lymphoid cells; and some of these release antibody. In such an ideal situation, equal amounts of the antibody from each plaque-forming cell (PFC) should diffuse into each indicator layer, thus providing 100 percent bilateral plaque correspondence in control assays. It is easy to imagine that trapped air bubbles, cellular debris, or even slight deviations from absolute flatness of the assay layers would result in pooling of the cell suspension at various sites and, depending on the random placement of any given PFC, favor expression of its plaque on the closer indicator layer while precluding its expression on the more distant layer. In practice, cell suspension filtration, air bubble extrusion, and the flexibility of the assay layers accommodate for these problems to a considerable extent; however, the average of 60 percent (*7*) bilateral plaque correspondence now obtained in control assays reflects the fact that ideal conditions have been approached rather than attained.

Direct quantitative comparison of the sensitivity of our assay with that of the standard Jerne plaque assay (*8*) is technically difficult because of the inherent loss of some immune cells at the time of air bubble extrusion. However, plots of plaque size distributions prepared both for γM and for γG replica plaques have configurations similar to those of standard PFC distributions (*3*). Moreover the replica plaque size distributions are complete including a few large, a few small, and many medium sized plaques. This indicates that most potential plaque-forming cells coming in contact with an assay layer were detected. In addition, since the ratio of γM and γG plaques for any given immune cell suspension is virtually the same, both by the standard Jerne assay and by the replica-imprint assay, there is reason to believe that neither γM nor γG plaque detection is selectively compensated.

Since in our work duplicate samples are lacking for about 40 percent of the plaque-forming cells detected, it would be premature to extend our conclusions to these PFC. We cannot rule out the possibility of a quiescent or latent period or the possibility that γM release may largely subside for a given cell before release of γG antibody begins. Nevertheless, our data demonstrate that very few, if any, rabbit spleen cells ordinarily release substantial quantities of

Table 1. Degree of γM-γG plaque pattern correspondence for rabbit cells releasing hemolysin, as determined by replica plating assays. D-D indicates that both original and imprint layers were developed directly for γM plaques; I-I refers to preparations in which both layers were developed indirectly for γG plaques; D-I represents preparations in which one layer was developed directly for γM and the other indirectly for γG plaques.

Immune cell pool	Original-imprint strip development	Plaques observed (No.)			Bilateral correspondence (%)	γM/γG
		Unilaterally on		Bilaterally		
		Original	Imprint			
		Fifth day after first immunization				
I	D-D	37	36	80	52	
I	D-D	28	32	152	72	
I	I-I	32	20	45	46	
I	D-I	215	51	2	0.75	4.21
I	D-I	290	46	2	0.59	6.30
		Sixth day after first immunization				
II	D-D	61	40	105	51	
II	I-I	19	13	51	61	
II	D-I	125	78	3	1.46	1.60
III	D-D	35	36	62	47	
III	I-I	12	9	42	67	
III	D-I	145	146	5	1.69	1.01
III	D-I	121	96	3	1.36	1.26
		Seventh day after first immunization				
IV	D-D*	60	57	123	51	
IV	D-D*	39	36	101	57	
IV	I-I*	18	23	92	69	
IV	D-I	421	374	14	1.73†	1.13
IV	D-I	406	260	12	1.77†	1.56
		Eighth day after first immunization				
V	D-D	45	70	107	48	
V	D-D	14	33	85	64	
V	I-I	36	34	93	57	
V	D-I	109	153	2	0.76	0.71
V	D-I	134	177	4	1.27	0.76
		Third day after fourth immunization				
VI	D-D	8	3	53	83	
VI	I-I*	7	23	49	62	
VI	D-I	11	100	1	0.89	0.11
VII	D-D	6	8	22	61	
VII	I-I*	18	17	31	47	
VII	D-I	12	47	1	1.69	0.26
VII	D-I	17	76	0	0	0.22

* Because of high plaque density or increased numbers of bubble artifacts (or both) in these photographs, smaller but representative portions of these photos were analyzed. † The apparent transient increase in the percentage of bilaterally correspondent plaques in these assays is ascribable to the increased plaque density which serves to increase the number of spuriously correspondent or bilaterally "coincident" plaques. The number of such "coincident" D-I plaques is a function of the product of direct times and indirect unilateral plaques.

both γM and γG hemolysins at any one time. Our findings are consonant with those of other related studies in which immunofluorescence was used to analyze the heavy chains in the globulins from nonviable lymphoid cells (*2*). But they are in contrast to a report by Nossal *et al.* (*1*) that viable immune cells from rat lymph nodes commonly contained both γM and γG antibody at the time of maximum class transition. There is evidence that neoplastic human lymphoid cells in tissue culture commonly contain and release more than one class of immunoglobulin heavy chain (*9*). A normal immune cell may contain two or more classes of antibody at a time, but at any given time it may attain the capacity to actively secrete significant amounts of only one molecular class.

An imprint replica procedure now permits simultaneous duplicate sampling of the active products of hundreds of individual cells in a single operation. It has been used to analyze hemolytically functional immunoglobulin class production by single cells, but it is equally applicable to the questions of possible double allotype or antibody specificity production by single cells. With minor modifications it could be used for populational analysis of gene expression in cells releasing any products which can be detected focally by chemical, radioautographic, enzymatic, or other means.

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5. The terms γM and γG are substituted throughout for the terms high hemolytic efficiency and low hemolytic efficiency, respectively, as a matter of convenience with the recognition that some γM antibody may be of low hemolytic efficiency (*6*). Use of these functional rather than structural criteria allows the additional test of whether γM antibodies of high and low hemolytic efficiency are likely to be produced and released in the rabbit by the same or separate cells.
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11. We thank W. Sewald and R. Nye for excellent photographic assistance.
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Metastasizing Mammary Carcinomas in Rats: Induction and Study of Their Immunogenicity

Abstract. *Widely metastasizing mammary adenocarcinomas were isolated from methylcholanthrene-fed inbred young adult female W/Fu rats that had been splenectomized or thymectomized, and from those that had been both splenectomized and thymectomized. These tumors are transplantable and maintain their metastasizing capacity. They are either nonimmunogenic or less immunogenic than the nonmetastasizing conventional methylcholathrene-induced mammary tumors, which are highly immunogenic.*

Unlike human solid tumors, their laboratory counterparts seldom metastasize. More specifically, the chemically induced mammary carcinomas in female rats usually do not metastasize spontaneously either in their autochthonous hosts or in syngeneic secondary hosts even after many generations of successive transplantations. Whether the tumor is hormone-responsive or autonomous, slow or fast growing, or with a long or short latency, there has been virtually no instance of genuine distant metastasis among the countless number of such tumors studied during the past several years in this laboratory (*1*).

An earlier observation (*2*) indicated that this absence or extreme rarity of metastasis in methylcholanthrene-induced mammary cancer was not due to the failure of these tumor cells to enter into the systemic circulation of the the host, for an overwhelming number of viable tumor cells were recovered from the arterial blood and the spleen of rats bearing a large primary (induced) or transplanted mammary adenocarcinoma, but rather was due to the tumor cells' being prevented from forming a cohesive mass in secondary sites. It was postulated, therefore, that the inability of the chemically induced rat mammary cancer cells to settle and establish a tumor colony in a distant site was caused by the potent specific antigenicity commonly found in the chemically induced murine tumors (*3*).

In a preliminary work (*4*), a widely metastasizing mammary adenocarcinoma was induced in a highly inbred W/Fu female rat. This rat was fed with 200 mg (20 mg $\times$ 10) of 3-methylcholanthrene and was splenectomized 1 day after the last feeding. When an early appearing, rapidly growing mammary carcinoma developed, it was surgically excised in order to keep the animal alive longer. This tumor was transplanted into syngeneic female rats and it grew progressively but did not produce any metastasis. However, another mammary adenocarcinoma developing 6 months after the excision of the earlier tumor (12 months after the carcinogen) was found to be metastatic. When this tumor (SMT-2) was transplanted, it spontaneously metastasized to lymph nodes, bones, liver, lungs, and other organs (Fig. 1). This metastasizing capacity seems to be an inherent character of the tumor cell, and the tumor metastasizes readily in syngeneic normal rats generations after the transplantation generation. The transplantation site (right inguinal mammary fat pad) and its procedure (injection of 0.1 ml of 1 : 1 tumor mince and Medium 199) were standardized throughout the experiment (*1*).