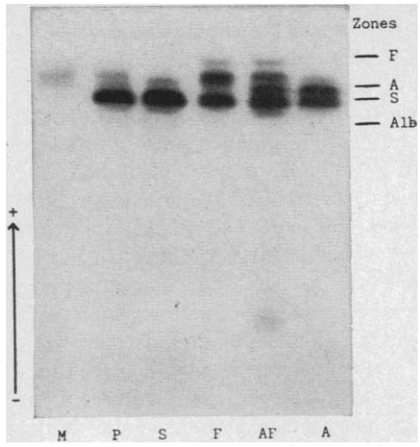

Fig. 1. Photograph of a gel slice stained in α-naphthyl acetate substrate solution and fixed in 50-percent methanol. This zymogram shows a system of rabbit serum esterases composed of six phenotypes labeled A, AF, F, S, P, and M. Atropinesterase acivity is limited to phenotypes A and AF and is believed to be localized in zone *A*. Cocainesterase activity is common to all phenotypes except M and is localized in zone *S*. *Alb* refers to the position of the albumin esterases which were clearly evident only when α-naphthol AS D and AS-LC acetates were used as substrates.

of the serum inhibited this color change throughout the 8-hour period. With cocainesterase-positive serum there were all degrees of color change ranging from those which progressed rapidly from red to light yellow to those which proceeded slowly from red to a reddish orange.

Among the 242 samples, there were 45 that failed to hydrolyze cocaine, and these nonreactors were restricted solely to the 45 rabbits of phenotype M. The only isozyme common to phenotypes A, AF, P, and S, and absent in phenotype M, is zone S (Fig. 1). Thus, it would appear that all the cocainesterase activity of rabbit serum is localized in isozyme S. Significantly, all 14 samples of phenotype AF and all 22 samples of phenotype S were classified as strong reactors, whereas weak reactions were observed only in phenotypes A, F, and P. These observations are consistent with the aforementioned genetic theory based on five alleles, because, according to that theory, all rabbits of phenotypes AF and S would be expected to possess a double dose of isozyme S, whereas some of the rabbits of phenotypes A, F, and P would be expected to be heterozygous for isozyme S and, therefore, would possess only a single dose of that isozyme.

With respect to both enzymes, three classes of rabbits were observed: those whose serums were positive for both enzymes (types A and AF), those whose serums were positive for cocainesterase but negative for atropinesterase (types F, P, and S), and those whose serums lacked both enzymes (type M). Ammon and Savelsberg (*1*) tested 22 samples of serum for both enzymes by means of a manometric method and, contrary to our observations, observed that some of the rabbits possessed atropinesterase but no cocainesterase. Although it is possible that such rabbits exist, we believe it is more likely that they overcorrected for nonenzymatic hydrolysis, thereby leading to the misclassification of some phenotypes.

In view of the foregoing observations, it is predictable that substrates may eventually be found that are hydrolyzed only by isozymes peculiar to phenotypes AF and F, namely, zone F and the intermediate zone of phenotype F. It is also predictable that the present results will lead to further experiments concerned with the biochemical, genetic (including ontogenetic and pharmacogenetic), and immunological aspects of atropinesterase and cocainesterase, especially as those two enzymes relate to the isozymes reported here. For example, it has been reported (*7*) that atropinesterase-negative rabbits produce isoprecipitins specific for atropinesterase when injected with serum from an atropinesterase-positive rabbit, and that the serum of rabbits which lack this enzyme contains no protein that is immunologically related to atropinesterase. The present results, along with those on the genetics of this six-phenotype system (*6*), would suggest that the isozymes of this system are closely related structurally and therefore should share common antigenic sites. With respect to hydrolytic properties, it has been reported that the enzyme in rabbit serum which hydrolyzes atropine is the same enzyme that hydrolyzes monoacetylmorphine, but Ellis (*8*) concluded that the activities of the plasma and liver of atropinesterase-positive rabbits on monoacetylmorphine, benzoylcholine, and atropine are due to three separate enzymes. We suspect that considerable tissue specificity of the isozyme patterns reported here will be revealed in subsequent studies and that such tissue specificity is responsible, at least in part, for the conflicting data. But irrespective of genetic, biochemical, and other considerations, we believe that knowledge of the six phenotypes described here will be helpful in clarifying the different responses of individuals to a variety of different drugs.

CLYDE STORMONT
YOSHIKO SUZUKI

Serology Laboratory,
University of California, Davis 95616

References and Notes

1. R. Ammon and W. Savelsberg, *Z. Physiol. Chem.* **284**, 135 (1949).
2. W. Kalow, *Pharmacogenetics* (Saunders, Philadelphia, 1962), pp. 53–55; *Ann. N.Y. Acad. Sci.* **151**, 694 (1968).
3. G. Werner, *Planta Med.* **9**, 293 (1951); *Arch. Exp. Pathol. Pharmakol.* **251**, 320 (1965).
4. R. L. Hunter and C. L. Markert, *Science* **125**, 1294 (1957).
5. E. M. Tucker, Y. Suzuki, C. Stormont, *Vox Sang.* **13**, 246 (1967).
6. C. Stormont and Y. Suzuki, in preparation.
7. F. Margolis and P. Feigelson, *Biochim. Biophys. Acta* **90**, 117 (1954).
8. S. Ellis, *J. Pharmacol. Exp. Ther.* **94**, 130 (1948).
9. We are especially indebted to Dr. Domenico Bernoco for his counsel and assistance in many phases of the tests, particularly those concerned with heat-treated samples of serum, and to Alan B. Combs for the conduct of the mydriatic tests.

18 August 1969; revised 22 October 1969

Influenza Virus Effects on Cell Membrane Proteins

Abstract. *During infection by influenza virus, viral proteins become firmly attached to (or part of) host cell plasma membrane, nuclear membrane, mitochondrial, and microsomal membranes. Purified virus particles contain less than 1 percent host cell protein. Virus envelope proteins completely replace host membrane proteins in those discrete spots on the plasma membrane from which progeny virions bud out.*

The cellular membranes of mammalian cells are composed of an elaborate array of proteins of widely different molecular weights (*1*), and the lipoprotein membranous envelope of arboviruses is composed of a single protein associated with phospholipid (*2*). We determined the protein composition of influenza virus envelope and of the membranes of cells infected by influenza virus because this virus matures by budding from the host cell plasma membrane (*3*). Laver (*4*) has shown that there are three major protein components in purified influenza virions, and he has partially characterized the major structural components of the virus membrane—the hemagglutinin and the neuraminidase. Using

ferritin-labeled antiviral antibody or anticellular antibody, Duc-Nguyen *et al.* (*5*) observed progressive accumulation of influenza virus antigens on the surface of infected cells and a progressive decrease in normal host antigens capable of reacting with antibody at the cell surface. Our study was carried out to determine whether virus membrane proteins are merely added to the many normal proteins already present in the plasma membrane of the infected cell, or whether they displace or substitute for the proteins of the plasma membrane to form a completely new virus envelope at those spots on the plasma membrane which bud out to form virus.

The NWS strain of mouse-neurotropic influenza virus was subjected to passage twice in mouse brain and twice in BHK21 hamster cells at low multiplicity to enhance its virulence to mammalian cells before working stocks were prepared in embryonated eggs. Cells of the MDCK (canine kidney) line were infected at an effective multiplicity of 10 plaque-forming units per cell. A plaque-forming unit in this cell line is equivalent to approximately 5 to 10 egg infectious doses. High multiplicity infection with this virus pool did not cause severe interference (Von Magnus effect) on first passage in these cells, and yields of more than 100 egg infectious doses per cell were obtained.

Electrophoretic separations of viral and cellular proteins were accomplished in 5 percent polyacrylamide gels containing 0.1 percent sodium dodecylsulfate (*1*, *6*). Cell and virus proteins were labeled in Eagle's minimum essential medium containing 2 percent dialyzed calf serum and H^3- or C^{14}-labeled tyrosine, phenylalanine, and valine. Protein concentration was determined by the Lowry method.

In agreement with Laver's original work on influenza virus (*4*) our electropherograms show at least three major proteins in purified virions. Furthermore, we have found that these are the only major proteins being synthesized in susceptible cells infected for several hours or more by NWS influenza virus (Fig. 1A). The three major proteins of purified virions and infected cells correspond, but there may be an additional virus protein as indicated by a shoulder on the middle peak (Fig. 1A). This virus inhibits protein synthesis in host cells, and substitutes virus-directed synthesis at a rate nearly equal that in uninfected cells (*7*).

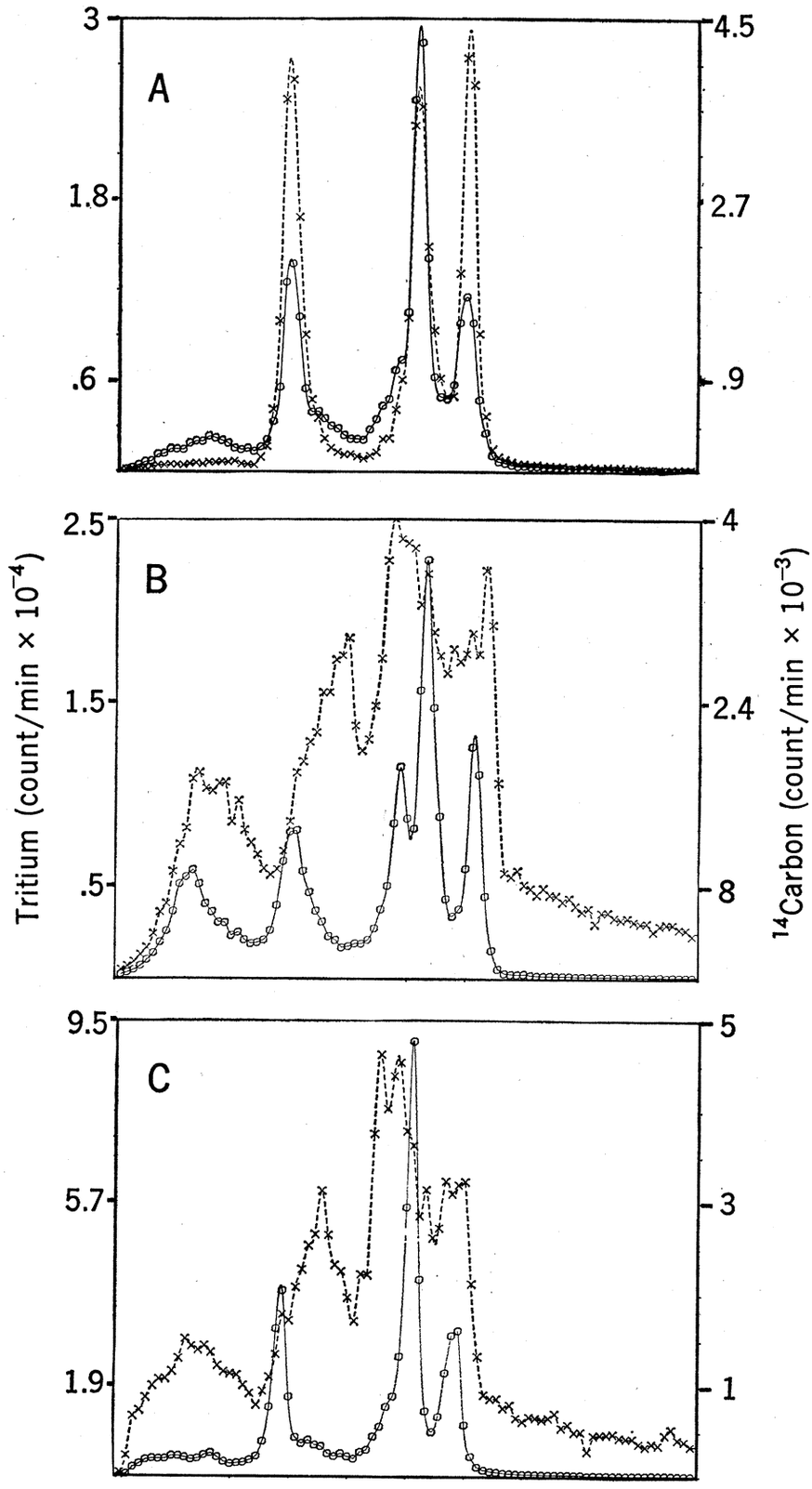

Fig. 1. Acrylamide gel electropherograms of labeled viral and cellular proteins. (A) Comparison of H^3-labeled proteins from purified influenza virions (crosses) and from intact cells labeled with C^{14} between 8 and 9 hours after infection with influenza virus (circles). (B) Comparison of washed C^{14}-labeled microsomal membranes from normal cells (crosses), and of washed microsomes that had been labeled while the cells were synthesizing viral proteins from 8 to 9 hours after infection (circles). (C) Same as (B) except mitochondria were isolated from normal and infected cells, washed, and their proteins analyzed. Note that five proteins appear in (B).

Microsomes and mitochondria of infected cells acquire virus proteins that are so firmly attached that they cannot be removed by repeated washing (Fig. 1, B and C). We have also observed a similar association of viral proteins with nuclear membranes and plasma membranes, so viral proteins appear to become part of, or at least nonspecifically attached to, most of the membranes of cells infected with influenza virus (*7*). When these membranes were extensively fragmented by high frequency sound so that about 30 percent of the host cell proteins were released in a soluble form, an equal proportion of virus protein was solubilized, and the remaining viral protein was greatly enriched in the largest virus polypeptide (the hemagglutinin) (*7*). Of course this could be merely nonspecific binding of relatively water-insoluble viral proteins to cellular membranes.

Because the virus membrane contains only a few proteins, and since it arises by budding from its host cell's plasma membrane (which has hundreds of proteins), we sought to determine whether the viral proteins are merely added to, or actually replace, the host membrane proteins. We labeled approximately 10^8 uninfected cells for 1 hour with 800 μc each of tritiated valine, phenylalanine, and tyrosine; then we washed the cells, stopped the labeling by addition of unlabeled amino acids, and infected them with NWS influenza virus. Between 4 and 7 hours after infection, we labeled continuously with C^{14}-valine, C^{14}-phenylalanine, and C^{14}-tyrosine. Fifteen hours after infection, virus particles were isolated from the medium; they were purified by adsorption and elution from red cells, ammonium sulfate precipitation, differential centrifugation, agarose column chromatography, and banding in density gradients of potassium sodium tartrate. Infected cells were also collected, washed free of medium, and various membrane fractions were isolated (*1*). A portion of these cells was not fractionated but was dissolved as whole cells in the sodium dodecylsulfate sample buffer (*1*) for electrophoresis and determination of specific activity. A gel electropherogram of these cells showed tritium distributed in the usual way among normal cell proteins of different molecular weights, whereas C^{14} was found primarily in virus-coded proteins (Fig. 2A). The specific activity of tritiated normal cell proteins in the cells was 10,700 count/min per microgram and in the purified virions it was only 530 count/min per microgram. The specific activity of the C^{14}-labeled viral proteins in the same cells was 1480 count/min per microgram and in the purified virions it was 2620 count/min per microgram. If there is a uniform distribution of tritium among all cell proteins, host protein constitutes less than 5 percent of the protein of purified virions. However, most of this tritiated protein is not host protein (gel electropherograms of the purified virions showed nearly all of the tritium as well as the C^{14} was present in viral polypeptides) (Fig. 2B). Apparently over 90 percent of the tritium in the virions was in viral proteins that became labeled with tritiated amino acids arising from turnover of tritiated host-cell proteins.

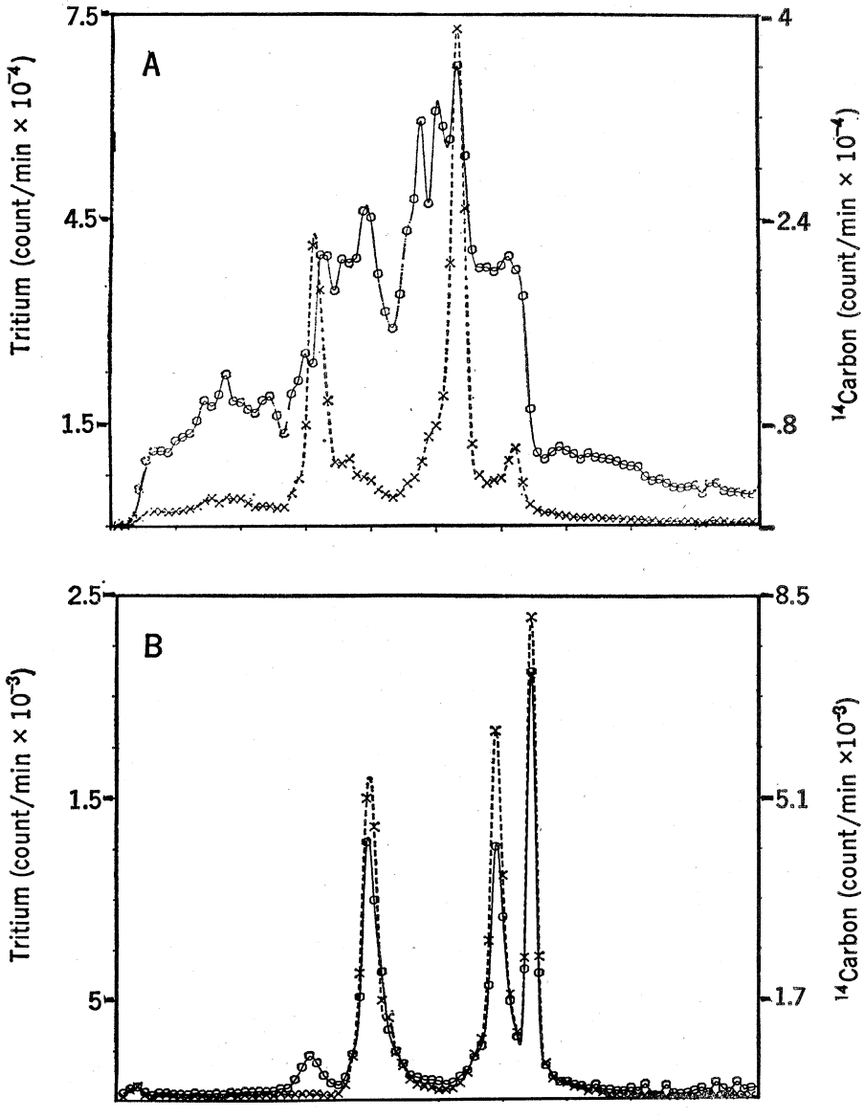

Fig. 2. Acrylamide gel electropherograms of proteins and viruses from cells labeled before and during influenza virus infection. (A) Comparison of proteins from whole cells labeled with tritiated amino acids 1 hour before influenza virus infection (circles), and then with C^{14}-labeled amino acids between 4 and 7 hours after infection (crosses). (B) Comparison of H^3- and C^{14}-labeled proteins of purified virions isolated 15 hours after infection from the medium of the same cells shown in (2A). The anode is on the left in all figures.

Thus, influenza virus membrane protein is virus-coded. If there is any membrane protein from the host in the virus envelope membrane, it is such a small amount that it could not be significant in membrane structure. Because this virus matures only when the internal nucleoprotein picks up the plasma membrane as an envelope which buds to the outside of the cell (*3, 5*) either all of the host protein of the plasma membrane is replaced by virus membrane protein, or it is completely re-

placed only in those patches which bud out to form virus. The latter must be the case, since the specific activity of the isolated plasma membranes (H^3, 10,080 count/min per microgram; C^{14}, 1820 count/min per microgram) shows most of the original host cell protein is retained along with new protein synthesized at the direction of virus. Of course, this would vary according to time of labeling, but there is much more host protein in the plasma membrane than in the virus which buds off from it.

If it is assumed that the first viral-membrane proteins to reach the plasma membrane act as nucleation points for a further aggregation of more of the same molecules either at random sites on the membrane, or perhaps at certain "weak" points where there is little or no host protein, then localized patches of membrane in which the only protein associated with the lipid components is virus-directed would appear. After these patches grow to a critical size they tend to attract and engulf nucleocapsid cores, and to bud away from the cell as part of a virion; thus, a cell's plasma membrane could form countless virions by budding without losing all of its normal membrane proteins.

This gel electrophoresis technique is capable of resolving the "structural proteins" of the rather simple influenza virus membrane as discrete peaks. This is in marked contrast to the hundreds of proteins observed by the same technique in normal cell membranes, and it is further evidence against the presence of one or a few major structural proteins in normal cell membranes (*1*).

JOHN J. HOLLAND
E. D. KIEHN

Department of Biology,
University of California at San Diego,
La Jolla 92037

References and Notes

1. E. D. Kiehn and J. J. Holland, *Proc. Nat. Acad. Sci. U.S.* **61**, 1370 (1968).
2. J. H. Strauss, Jr., B. N. Burge, E. R. P. Pfefferkorn, J. E. Darnell, Jr., *ibid.* **59**, 533 (1968); F. H. Yin and R. S. Lockart, Jr., *J. Virol.* **2**, 728 (1968).
3. L. Hoyle, *J. Hyg.* **52**, 180 (1954); C. Morgan, H. M. Rose, D. H. Moore, *J. Exp. Med.* **104**, 171 (1956).
4. W. G. Laver, *J. Mol. Biol.* **9**, 109 (1964).
5. H. Duc-Nguyen, H. M. Rose, C. Morgan, *Virology* **28**, 404 (1966).
6. J. V. Maizel, *Science* **151**, 988 (19[illegible] D. F. Summers, J. V. Maizel, J. E. Darnell, *Proc. Nat. Acad. Sci. U.S.* **54**, 505 (1965); *Virology* **31**, 427 (1967); J. V. Maizel, B. A. Phillips, D. R. Summers, *ibid.* **32**, 692 (1967).
7. J. J. Holland, E. D. Kiehn, E. Penhoet, in preparation.
8. We thank E. Bussey for technical assistance. Supported by NIH grant CA-10802.

8 September 1969; revised 22 October 1969

Polyinosinic-Polycytidylic Acid Inhibits Chemically Induced Tumorigenesis in Mouse Skin

Abstract. *Polyinosinic-polycytidylic acid administered intraperitoneally inhibits (i) the formation of skin tumors induced by a single topical application of 9,10-dimethylbenzanthracene followed by weekly applications of croton oil and (ii) the formation of skin tumors induced by a single large application of 9,10-dimethylbenzanthracene.*

A single small dose of 9,10-dimethylbenzanthracene (DMBA) topically applied to mouse skin initiates tumorigenesis (*1*). The initiated foci may then be promoted to the visible tumor stage by the weekly application of a promoting agent such as croton oil (*1, 2*). Tumorigenesis may also be induced by a single large topical application of DMBA with no subsequent treatment with croton oil. The molecular mechanism of tumorigenesis is not known. The chemical carcinogen may directly transform normal cells to the tumor state, may activate latent tumor viruses, or may permit the development of either preexisting or carcinogen-transformed tumor clones which are normally suppressed (*3, 4*). The latter may relate to the role of immunological mechanisms in tumorigenesis. One theory of carcinogenesis suggests that tumor cell clones are normally inhibited from further development by immunological processes (*4*). A failure of normal immunological suppression would result in the development and appearance of gross tumors. The chemical carcinogen or promoter may, through immunosuppressive activity or by increasing the number of tumor cells formed, allow the formation of gross tumors. This may occur when normal immunological exclusion of tumor cells is overcome.

The synthetic double-stranded RNA, polyinosinic-polycytidylic acid (poly I • poly C) induces cells to synthesize interferon and to develop strong resistance to virus replication (*5*). In addition, synthetic polynucleotides enhance the immunological capability of the host as shown by an increase in circulating antibodies (*6*). Also, poly I • poly C inhibits the growth of several transplanted tumors (*7*). We now re-

Table 1. Effect of poly I • poly C on skin tumorigenesis at 11 to 12 weeks initiated by DMBA and promoted by croton oil. Each group consisted of 35 male Swiss mice weighing 18 to 22 g. The initial treatment was given in week 1; subsequent treatment was given after week 1. In experiment 1, 25 μg of DMBA in 0.2 ml of acetone was applied topically to the backs of cleanly shaven mice. Poly I • poly C (200 μg/0.2 ml per mouse) was given to group 3 on days 1, 2, 4, 6, and 9. The DMBA was given on day 2. In group 5 six doses of poly I • poly C (200 μg per 0.2 ml) was given on days 1, 3, 5, 7, 9, and 11. The DMBA was given on day 5. Mice in groups 5 and 6 received subsequent injections of 0.2 ml of poly I • poly C thrice weekly. All mice received topical applications of 0.2 ml of 1 percent croton oil once a week, starting 1 week after DMBA was given. All mice not given poly I • poly C received saline. In experiment 2, group 2 received five injections of 100 μg of poly I • poly C per 0.1 ml on days 1, 3, 4, 6, and 8. Group 4 received 100 μg of poly I • poly C per 0.1 ml thrice weekly, starting 1 week after DMBA was given. All mice received croton oil treatment as in experiment 1. All mice not given poly I • poly C received saline.

Group	Treatment		Survivors (No.)	Mice with tumors (No.)	Total tumors (No.)	Tumors per mouse (av. No.)	Percent of DMBA control
	Initial	Subsequent					
			Experiment 1A				
1	Acetone	None	31	0	0	0	0
2	DMBA	None	32	18	122	3.8	100
3	DMBA + Poly I • poly C	None	31	16	72	2.3	61
			Experiment 1B				
4	DMBA	None	30	17	102	3.4	100
5	DMBA + Poly I • poly C	Poly I • poly C	18	2	2	0.1	3
6	DMBA	Poly I • poly C	22	5	7	0.3	9
			Experiment 2A				
1	DMBA	None	30	28	154	5.1	100
2	DMBA + Poly I • poly C	None	28	25	166	5.9	116
			Experiment 2B				
3	DMBA	None	28	24	161	5.8	100
4	DMBA	Poly I • poly C	19	9	15	0.8	14