Ph.D.
ABSTRACT

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STUDIES ON MODES OF ACTION OF ANTICELLULAR ANTIBODIES

An in vitro system containing a rabbit serum medium-adapted subline of L5178Y cells and rabbit antiserum was used to study the effects of antibodies, in the absence of complement, on cells in culture.

Heat-inactivated antiserum inhibits cell growth and colony formation in soft agar, and kills the cells. These actions occur slowly and metabolic events, in sequence; that is, RNA synthesis is inhibited after 4 hr, but the effects on the rates of protein and DNA syntheses become demonstrable only after 6 to 8, and 8 hr, respectively. Maximal inhibition can be obtained after 24 to 48 hr.

These effects together with the apparent involvement of energy generating systems suggest that some indirect mechanism, triggered as a result of primary antigen-antibody reactions on the cells surface, is responsible. An indirect mode of action mediated by second messengers, such as cyclic AMP, is proposed.

STUDIES ON MODES OF ACTION OF ANTICELLULAR ANTIBODIES

T. J. YANG

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by

TSU-JU YANG

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Department of Microbiology and Immunology
McGill University
Montreal

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CHAPTER I. INTRODUCTION AND REVIEW OF LITERATURE

Immunological reactions which cause alteration or damage of tissue cells are many and include both indirect and direct effects (Wissler, 1962). Indirect reactions are those in which the antibody is directed neither against the tissue cells nor their components (i.e., acute anaphylaxis, the Arthus reaction and serum sickness). Direct reactions are those in which specific antibodies are involved against tissue cells and their components (i.e., tumor, auto-immune states and, possibly, the rejection of allografts (Wissler, 1962; Stetson, 1963)).

In general, each of these immuno-pathological processes involves the response of blood and lymph vessels as well as the inflamatory cells; and in some instances, at least, they are brought about by the release of mediators of inflammation, including esterases, histamine and other stored permeability factors. As a result these complicated and variable effects of cytotoxic antibodies in the intact animals have prevented an understanding of their exact action on tissue cells. It since has been found, however, that tissue culture affords a method for the study of cytotoxic antibodies in which their direct effects can be distinguished from cellular defense, vascular reaction, clotting mechanisms, and other mediators of resistance and homeostasis that exist in vivo. This system will allow the direct observation of cells in relatively uninjured state for a longer period of time than was formerly possible.

Lambert and Hanes in 1911 were the first to utilize the

tissue culture technique for this purpose. They reported that rat sarcoma would not grow in plasmacoagulum from guinea pigs immunized with rat sarcoma tissue. Since then a number of reports have appeared on the in vitro effect of cytotoxic antibodies, Lambert (1914), Lumsden and co-workers (1931) measured the ability of antibody to inhibit the outgrowth of explants and, in 1929, Niven made the first study on the nature of the damage inflicted upon growing cultures of cells by cytotoxic antibodies.

Although many studies have been made on both the theoretical and the practical aspects of cytolysis, and remarkable advances have been made in complement research, most of these experiments were concerned with morphological changes of the cells in culture. Few observations have been made on the exact nature of the injury inflicted upon cells by the antiserum or on the accompanying metabolic events.

The main purpose of the work was to investigate immunity to transplantable tumors, that is, to find whether cytotoxic antibodies were specific for tumor cells. In the search for cancer-specific antibodies, the indicators of activity were the destruction or death of cell and the prevention of cell " outwandering " and growth (Lumsden, 1931). Similar work was done on body cells in general, and authors stressed that the cytotoxic action was organ or species specific (Lambert, 1914; Verne & Oberling, 1932; Harris, 1948; Kite et al., 1967).

The first clear evidence about the nature of the damage to the cell came from Green and co-workers (1959a, b) who studied the

cytotoxic action of rabbit antibody and complement on Krebs ascites tumor cells in vitro. They observed that, within a few minutes, most of the smaller intracellular ions and molecules such as potassium, free amino acids, and ribonucleotides were lost from the cell together with a large but variable proportion of the intracellular RNA and protein. These cellular components escaped through a cell membrane which appeared, in phase and electron microscopic examinations, to be altered (although unruptured) and still able to discriminate to some degree against the passage of larger molecules.

The specific cytotoxic action of antibodies on cells has been well established (Table 1) and it is generally believed that complement is needed for this action. Detailed work carried out in the laboratory of Goldberg (Goldberg, 1963) has clearly shown its mechanism to be very similar to that of red blood cell immune hemolysis. As can be seen in the review of the literature (Table 1) most of the observations made were for the period used for conventional complement-mediated immune hemolysis system (i.e., 30 min to 2 hr). The reactions in this system usually occur too quickly for the study of metabolic sequences. Consequently, most problems relating to the ability of nucleated cells to " neutralize " and to repair or compensate for moderate amounts of immune damage still remain to be solved (Harris, 1948; Winn, 1965). In order to study the effects of antibodies on nucleated mammalian cells in greater detail and to establish what is due to the binding of antibodies and what is dependent on the lytic activity of the complement, a system which would allow sequential

Table 1 : Effects of Anticellular Antibodies on Cells in Culture in the Presence of Complement

Antiserum	Cells	Effects	Reference
Rat anti-mouse sarcoma & guinea pig anti-rat sarcoma	Mouse sarcoma & rat sarcoma cells	Inhibition of initiation of growth; 1 to several days.	Lambert & Hanes, 1911.
Rabbit anti-human tonsil lymphocytes, & rat thymus cells	Rat thymus cells	Trypan blue dye uptake; morphological changes; 35 min.	Pappenheimer, 1917.
Rabbit anti-mouse embryo tissue	Mouse heart fibroblasts	Inhibition of cell emigration; death of actively growing cells; 35 min.	Niven, 1929.
Rat anti-chick heart	Chick heart explants	Immediate drastic effects; 15 min to 6 hr.	Harris, 1948.
Rabbit anti-Brown-Pearce carcinoma cells	Brown-Pearce carcinoma cells	Cellular changes & cell death; within 2 hr.	Kalfayan & Kidd, 1953.
Guinea pig anti-mouse mammary carcinoma	Mouse mammary carcinoma explants	Cell death, 3 to 6 hr, 16 hr.	Imagawa et al., 1954
Rabbit anti-HeLa cells	HeLa cells	Morphological changes, " blisters ", cell death; a few hrs to several days.	Mountain, 1955.

Table 1 : Effects of Anticellular Antibodies on Cells in Culture in the Presence of Complement (continued)

Antiserum	Cells	Effects	Reference
Rabbit anti-Ehrlich ascites cells	Ehrlich ascites cells	Immediate cessation of respiration; unable to utilize endogenous substrate or exogenous glucose, cell death.	Flax, 1956.
Rabbit anti-Ehrlich ascites tumor cells	Ehrlich ascites cells	Inhibition of nucleic acid & protein syntheses; loss of cell protein & RNA; 90 min.	Colter et al., 1956.
Rabbit & chicken anti-HeLa cells	HeLa cells & normal human cells	Cell injury & destruction; 20 min to 2 hr. Cinematographic study.	Miller & Hsu, 1956.
Guinea pig anti-chicken embryo heart	Chicken embryo heart fibroblsts	Morphological changes, & cell death; 15 to 90 min.	Latta & Kutsakis, 1957.
Rabbit anti-HeLa cells	HeLa cells	Irreversible cytotoxic changes within 2 hr.	Goldstein & Myrvik, 1958
Rabbit anti-Krebs-2 ascites tumor cells	Krebs-2 ascites tumor cells	Phase & electron microscopic study; distinctive alteration in cellular surface membrane; subsequent cytoplasmic changes.	Goldberg & Green, 1959.

Table 1 : Effects of Anticellular Antibodies on Cells in Culture in the Presence of Complement (continued)

Antiserum	Cells	Effects	Reference
Rabbit anti-Krebs-2 ascites tumor cells	Krebs-2 ascites tumor cells	Loss of free amino acids, ribonucleotides, K^+, RNA & cell protein within a few min. No changes detectable in DNA.	Green et al., 1959b.
Rat & rabbit anti-Ehrlich ascites carcinoma cells	Ehrlich ascites carcinoma cells	Inhibition of respiration, uptake & incorporation of ^{14}C-l-glycine, exudation of cellular materials; cytological changes; 1 to 2 hr.	Bickis et al., 1959.
Rabbit anti-human amnion cells	Human amnion cells	Inhibition of acid prodution in medium; increase in trypan blue uptake.	Ross & Lepow, 1960.
Rabbit anti-human connective tissue	HeLa cells	Cytotoxic effects start from 6 min.	Hiramoto et al., 1960
Guinea pig anti-Chang's conjunctiva cells	Chang's conjunctiva cells	Cytotoxicity most effective during the stage of cytoplasmic cell division, i.e., between mitosis & Gl-phase.	Bucen et al., 1966.
Rabbit anti-HeLa & L cells	HeLa & L cells	Cytotoxic effects.	Bartholomew et al., 1967.

Table 1 : Effects of Anticellular Antibodies on Cells in Culture in the Presence of Complement (continued)

Antiserum	Cells	Effects	Reference
Rabbit anti-Ehrlich ascites tumor cells	Ehrlich ascites tumor cells	Leakage of cell constituents.	Nungester et al., 1969.

follow up studies was essential. Furthermore, certain evidence and some casual obervations suggested that longer experimental periods were needed to demonstrate cytotoxic effects in the absence of complement. As early as 1931, for instance, Lumsden noted that " in the absence of complement, antibodies cause agglutination of cells and not infrequently a precipitation reaction and gradual death are observed ". Using experiments ranging from 24 hr to 5 days, Liu, McCrory and Flick (1957) found cytotoxic action of anti-rat kidney antibody on rat renal tissue cultures. This action caused the loss of at least 50 % of the cells and microscopic alterations in those remaining. Lippman, Cameron and Campbell (1950) also reported the inhibitory effect of ammonium sulphate precipitated immune globulin on cells in culture. Habel et al. (1958) and Quersin-Thiry (1958) reported independently that the cytopathic effect of certain enteroviruses in tissue culture was inhibited by antiserum prepared against the cells of the culture. Holland and McLaren (1959) found that anti-HeLa serum strongly inhibited plaque formation by type 1 poliovirus in HeLa cells, ERK-1, Minn. EE and monkey kidney cells, both confirming and extending the above results. Timbury (1962) also reported that serum prepared against amnion cells had strong cytotoxic effects on amnion cell monolayers and that in a sub-cytotoxic dilution (1:32) strongly inhibited plaque formation by ECHO viruses and by Coxsackie virus type A9. He mentioned that the inhibition was not due to the specific neutralizing effect on the virus and that the reaction did not require the presence of complement. Quersin-Thiry

(1958), Holland & McLaren (1959) and Timbury (1962) suggested that the inhibitory effects of anticellular antibodies on viruses may be due to specific combination with antigenic receptor sites on the cell surface or, alternatively, that viral receptors may be partially covered by antibodies reacting with neighbouring antigenic determinants. On the otherhand, Habel et al. (1958) have suggested that metabolic distrubance of host cells by antibodies is a likely cause because anticellular serum inhibited the viral cytopathic effect when added in the latent period of infection. Habel concluded that the blocking of viral adsorption sites was not the sole mechanism of inhibition and that the influence of antiserum resulted from a general cellular inhibition. Timbury (1963), himself, later confirmed this suggestion by showing that the anti-human amnion cell serum inhibited the intracellular replication of Coxsackie A9 virus in amnion cells but did not affect either the viral adsorption or eclipse period. The formation of infective centers and intracellular virus was greatly reduced, although not completely suppressed, in suspensions of cells treated with antibodies.

In the above-mentioned reports antibody alone, in the absence of complement, seemed to be harmful to the cells in culture although more controlled conditions were needed before a definite conclusion could be made. In most of the experiments, for example, ammonium sulphate precipitated gamma globulin from non-heated antiserum had been used with or without culture medium containing fresh serum or embryo extracts (Lippman, 1950; Ehrlich & Halbert, 1961; Ehrlich, Halbert &

Manski, 1962).

In many of the experiments, the tissue explants used contained stromal cells in addition to endothelial or epithelial cells and all of these contribute components of complement to the culture or cause their production (Thorbecke et al., 1965). Although some workers were cautious enough to rinse the cultures with balanced salt solutions before adding antisera, they possibly could have introduced another factor by previous absorption of the antisera and the non-immune normal sera with lyophilized pooled normal serum; the absorption was to eliminate the non-tissue antibodies for all tests (Basset et al., 1957; Ehrlich & Halbert, 1961; Ehrlich et al., 1962). Moreover, rinsing the antiserum-reacted cells before further culture in tissue culture medium requires careful control because it is well known that it is difficult to wash the cell surface free of antibodies (Kite et al. (1967) have demonstrated carry over of serum in tissue culture medium for three generation). So, in many of the tissue culture systems used the workers may not have completely excluded the heat-labile components in their attempts to evaluate the effects of heated antiserum. This may be one reason that some of them described a " blebbing " of the cytoplasm which was similar to responses noted by others in complement-containing systems. Literature concerning the non-complement mediated systems is listed in Table 2.

The purposes of the present study were several. Firstly, to investigate the effects of antibodies on the nucleated mammalian cells

Table 2 : Effects of Anticellular Antibodies on Cells in Culture in the Absence of Added Complement

Antiserum	Cells	Effects	Reference
Rabbit anti-mouse embryo tissue	Mouse fibroblasts	Delayed cytotoxic action, complete death at 15 to 30 hrs.	Niven, 1929.
Sheep anti-mouse & human cancers, and rabbit anti-mouse cancer	Mouse sarcoma (S37) & cancer (M63) cells	Gradual death.	Lumsden, 1931.
Rabbit anti-rat kidney	Explants of rat & chicken heart muscle & kidney	No growth.	Lippman et al., 1950.
Rabbit anti-HeLa cells	HeLa cells	Large clumps of cells rounding up & peeling off the wall; a few hrs to 5 days.	Mountain, 1955.
Guinea pig anti-chicken embryo heart	Chicken embryo heart fibroblasts	No toxic activity over the 3 hr observation period	Latta & Kutsakis, 1957.
Rabbit anti-rat kidney	Rat kidney cells	Cytopathogenic effect within 5 days.	Liu et al., 1957.
Rabbit anti-human skin & placenta	Human skin explants with outgrowth	Cytopathogenic effect within 1 week.	Basset et al., 1957.
Rabbit anti-HeLa cells	HeLa cells	Marked depression of growth rate	Goldstein & Myrvik, 1958.

Table 2 : Effects of Anticellular Antibodies on Cells in Culture in the Absence of Added Complement (continued)

Antiserum	Cells	Effects	Reference
Rabbit anti-HeLa & monkey kidney	HeLa & monkey kidney cells	Rounding & agglutination after several hrs. Protected the cells from viral cytopathogenic effect.	Quersin-Thiry, 1958.
Rabbit anti-Krebs-2 ascites tumor cells	Krebs-2 ascites tumor cells	Cell surface membrane change, invagination & interdigitation of projections from the surfaces of apposed cells	Goldberg & Green, 1959.
Duck anti-rabbit cornea (absorbed with normal rabbit serum)	Rabbit cornea explants	Cytological changes after 3 to 6 hrs	Ehrlich & Halbert, 1961
Duck anti-rabbit lens	Rabbit lens explants	Cytotoxic after 3 to 20 hrs.	Ehrlich et al., 1962.
Rabbit anti-human amniotic membranes	Human amnion cells	High concentration: cytotoxic; low concentration: inhibition of virus growth & virus cytopathogenic effect; after 24 hrs.	Timbury, 1962.
Rabbit anti-HeLa & L cells	HeLa & L cells	Cytopathogenic effect after 1 to 5 days.	Bartholmew et al., 1967.

Table 2 : Effects of Anticellular Antibodies on Cells in Culture in the Absence of Added Complement (continued)

Antiserum	Cells	Effects	Reference
Rabbit anti-Yoshida ascites hepatoma	Yoshida hepatoma cell & its cell-free system	Depression of aerobic & anaerobic incorporation of ^{14}C-l-glycine into protein.	Marsilii & Chiarugi, 1968.
Rabbit anti-HEP-2 cells	HEP-2 cells	Echovirus plaque formation inhibited	Timbury, 1969.
Horse & goat anti-human lymphocytes	Mixed lymphocyte culture	Inhibition & stimulation of mixed lymphocyte reactions, ^{3}H-thymidine incorporation.	Bach & Bach, 1970.
Duck anti-rabbit cornea	Rabbit corneal epithelium explants	No change at 90 min; cytotoxic effects after 3 to 6 hrs, showing rounding of cells & disorganization of the cell sheet.	Manski & Ehrlich, 1970.

in the absence of complement but under more controlled systems than have been previously used. Secondly, to see if and how antibodies affect cell metabolism and growth in the absence of complement. And, finally, to establish the sequence of metabolic events caused by antibodies alone for, when complement is present, it has been impossible to differentiate between the effects due to the binding of antibodies and those dependent on the lytic activity of the complement.

CHAPTER II. EXPERIMENTS AND DISCUSSION OF RESULTS

Part I. Effects of Heat-inactivated Antisera on the Biochemical Activities of Mouse Leukemia L5178YR Cells in Culture.[1]

1. Introduction

The cytotoxic effect of antibodies on nucleated mammalian cells treated *in vitro* has generally been shown to require the presence of complement (Gorer & O'Gorman, 1956; Wissler, 1962; Bitensky, 1963; Goldberg, 1963; Winn, 1965). In the absence of complement the cells have been reported to appear normal when viewed by phase-contrast microscopy (Goldberg & Green, 1959); they retain their capacity to exclude trypan blue (Gorer & O'Gorman, 1956); and the selective permeability, chemical constitution and metabolic properties of the cells appeared not to be significantly affected (Flax, 1956; Bickis, Quastel & Vas, 1959; Green *et al.*, 1959b; Johnstone & Vas, 1961). Therefore, antibody alone is believed not to interfere with the structural and functional integrity of cell membranes (Müller-Eberhard, 1968).

In view of the fact that most of the experiments on the effect of antibodies on cells were done for a period of only 2 hr or less, and that most lymphotoxic agents such as cortisone start to show a slight killing effect after 5 hr and a maximal effect after 24 hr (Trowell, 1965), it was decided to reinvestigate the effect of antibodies on cells for longer periods of time.

The results of this work show that antiserum alone is inhibi-

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(1) The result has been published in Cancer Research 30:1231-1235, 1970.

tory to cell metabolism and growth and has cytocidal activity starting at 6 hr and reaching a maximum 24 to 48 hr after its addition to tissue culture. This suggests, as does Trowell's explanation for lymphocyte poisons (Trowell, 1965), that the failures of some workers to find any effect from antisera may, in some cases, have been due to the fact that they did not observe the cells for long enough periods.

2. Materials and Methods

a. L5178YR Cells

L5178Y mouse leukemia cells (Fischer & Sartorelli, 1964), obtained from Dr. R. Momparlar, McGill University Cancer Research Unit, were adapted gradually from medium containing horse serum to medium containing rabbit serum. They were designated as L5178YR subline. The cells had been passaged more than 24 times in complete rabbit serum medium before use in experiments. (The cells at present are in the 231st passage.) Fischer's medium for leukemic cells of mice was obtained from Grand Island Biological Co. (Grand Island, N.Y., U.S.A.) and was sterilized by filtering through a sintered glass filter. The medium contained 10 % heat-inactivated normal rabbit serum which had been prefiltered through a washed Millipore filter (Cahn, 1967). The cells contained no demonstrable viral particles or Mycoplasma. The cultures were maintained by routine subculture every 2 to 3 days.

b. Production of Antisera

After pre-immunization bleeding, adult male New Zealand white rabbits were immunized intravenously with 2

pulses of 2.5-3.5 x 10^8 viable L5178YR cells which had been washed 3 times with the Fischer's medium without serum and injected according to the schedule of Levey and Medawar (Levey & Medawar, 1966). L5178YR cells were used for the production of antisera in rabbits to avoid production of antibodies against horse serum components present in the medium of the original L5178Y cells. Furthermore, the possibility of an effect by allo-antibodies was excluded in preliminary experiments by testing the antisera with the L5178Y cells grown in the original medium containing horse serum. There was no difference in effect. Sera were collected aseptically and stored at -70 C until use. They were heat-inactivated (56C, 30 min) just before use. Sera for metabolic studies were selected on the basis of agglutinating and cytotoxic titers. The cytotoxic titer had to exceed the agglutinating titer to exclude the influence of clumping on the experiments.

c. Culture Conditions

Cultures which received an inoculum to provide 1 x 10^5 viable cells per ml and were incubated for a period of 48 hr in stationary Erlenmeyer flasks served as seed cultures. At this stage, they were near the end of the logarithmic phase of reproductive activity and had a population density of about 1 x 10^6 viable cells per ml (The generation time was 13 to 18 hr with a mean generation time of 14.5 hr). The cultures were adjusted to provide 5 x 10^5 viable cells per ml for radioisotope labeling studies and 1 x 10^5 viable cells per ml for cell examination.

From a single cell pool five ml cultures were set up, in

Bellco tissue culture tubes. Antisera and one of the substrates to be tested[2] were added and the tubes stoppered with white rubber stoppers. Experiments of 6 hr duration or less were carried out in a 37 C water bath in an upright position while those longer than 6 hr were placed into an incubator in a slanted position, in order to obtain maximal air space in the tube. Metabolic studies were made at 1/2, 1, 2, 4, 6, 24, 48, 72 and 96 hr after the start of the experiment. Viable and total cell counts were made at each sampling.

d. Metabolic Studies

The metabolic studies of the cells treated with antisera were based on the methods described by Bickis et al. (1959). At the above stated times, 5 uc of ^{14}C-2-glycine (Sp. Act. 1.12mc/m mole, Merck Sharp & Dohme of Canada Ltd., Montreal) in 0.2 ml of Fischer's medium (without serum) was added to each culture. After incubation for a further 60 min in the 37 C water bath, the cell suspensions were transferred to conical centrifuge tubes. Three-tenths ml of each cell suspension was taken for cell counts; the rest was centrifuged, and then washed 4 times with Fischer's medium (without serum) at room temperature (Piperno & Oxender, 1968). After the final wash, the cells were re-suspended in 5 ml of medium and divided into two equal portions and centrifuged. One portion was

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(2) 200 mM solutions of sodium succinate, sodium malonate, glucose, sodium malate and nicotinamide. Each was passed through a sintered glass filter and was added to the test system in amounts stated.

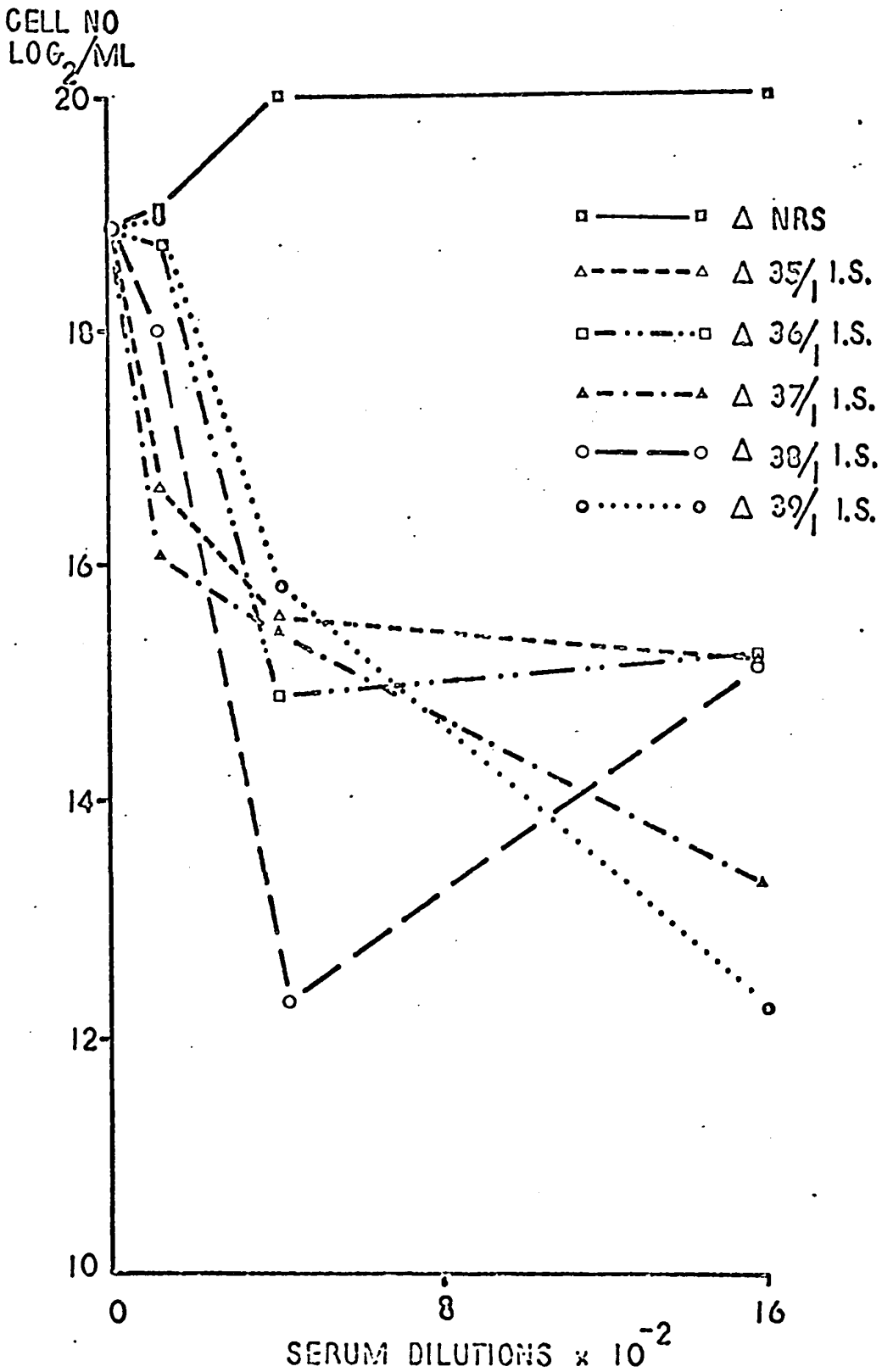
used for the determination of uptake and accumulation of the labeled amino acid in the cell and the other sample for the determination of incorporation of labeled amino acid into cell proteins. To determing the uptake, 2.5 ml of 95 % ethanol was added to the cell button and left for at least 2 hr at room temperature to break the cells and precipitate their protein. It was then centrifuged, and a half of the supernatant was added to 15 ml of Bray's scintillation fluid for radioactive assay. To determine the incorporation of the labeled amino acid into cell protein, the cell button was treated with 6 % trichloroacetic acid (TCA) in the refrigerator overnight and the precipitate extracted with hot TCA at 90 C for 15 min to remove nucleic acids. The residue was then washed 3 times with cold TCA to remove acid-soluble, radioactive components. The lipids were removed by washing first with 95 % ethanol at 60 C for 10 min, then with 95 % ethanol at room temperature, next with a mixture of 95 % ethanol and ether (3:1) and finally, with ether. The residue was dissolved in 1 ml Hyamin (hydroxide of Hyamine 10-X, Packard Instrument Co. Inc., Downers Grove, Illinois) and half of it was added to Bray's scintillation fluid. The radioactivity was assayed in a Packard Tri-carb liquid scintillation spectrometer Model 3375 (Packard Instrument Co. Inc., La Grange, Illinois).

3. Results

a. The Effect of Antibodies on the Multiplication of the Cells

As shown in Figure 1, the dose-response curves

Figure 1. The effects of various antisera on the viable cell counts of L5178YR cells in culture. Cell inoculum at 0 hr, 1 x 10^5 viable cells/ml; duration of experiment, 48 hr. ΔNRS, heat-inactivated normal rabbit serum; Δn/1 I.S., batch of heat-inactivated immune rabbit serum.


CELL NO
LOG2/ML
20
18
16
14
12
10
0
8
16
SERUM DILUTIONS x 10-2
Δ NRS
Δ 35/1 I.S.
Δ 36/1 I.S.
Δ 37/1 I.S.
Δ 38/1 I.S.
Δ 39/1 I.S.

of individual antisera differ greatly.

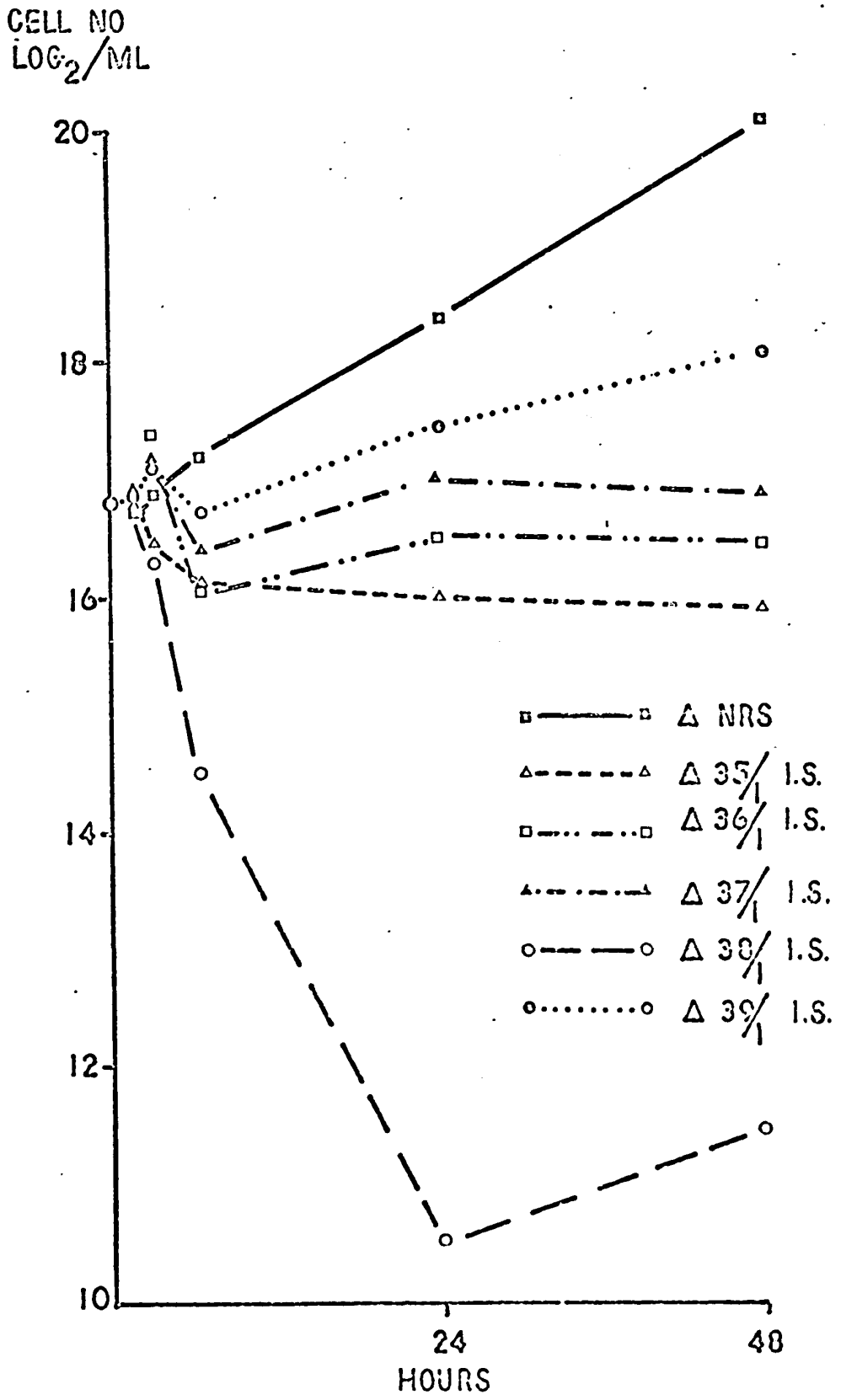
Figure 2 demonstrates that the inhibitory or killing effect of the antisera which started after 6 hr and reached its maximum after 24 to 48 hr. No harmful effects of the antisera were demonstrable at 1, 2 and 4 hr. The decrease in total cell counts, i.e., viable plus dead cells, was even slower and the initial cause of cell death seemed not to be due to cell lysis as in the complement-mediated system. After 24 to 48 hr, and even at the longest observation period of 96 hr, there was always a population of surviving cells. In general, these cells were much larger in size (diameter 22.35±1.17u) than normal stationary cells (diameter 15.66±1.39u). In addition, the proportion of very large cells (diameter 30.92±1.80u), which constituted 0.5-1.5 % of the cells in the initial cultures, was increased.

It was interesting to note that with some antisera there was a transient increase in cell number, 2 hr after the start of the experiments.

b. Studies on the Metabolism of Antibody Treated Cells

As shown in Figure 3, both ^{14}C-2-glycine uptake and incorporation was inhibited by antibody in the cultures. When the results were expressed in terms of 10^6 viable cells it can be seen that the incorporation of glycine was doubled although the amino acid uptake had not increased at the same rate (Figure 4). There was a transient stimulation of uptake and incorporation of ^{14}C-2-glycine at 2 hr paralleling the increase in cell counts (Figures 2 & 3).

Figure 2. Time effects of various antisera on the viable cell counts of L5178YR cells in culture. Cell inoculum at 0 hr, 1×10^5 viable cells/ml; antiserum dilution, 4×10^{-2}. Δ NRS, heat-inactivated normal rabbit serum; Δ n/l I.S., batch of heat-inactivated immune rabbit serum.


CELL NO
LOG2/ML
20
18
16
14
12
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24
48
HOURS
Δ NRS
Δ 35/1 I.S.
Δ 36/1 I.S.
Δ 37/1 I.S.
Δ 38/1 I.S.
Δ 39/1 I.S.

Figure 3. Time effects of antiserum 37/1 on the uptake and incorporation of ^{14}C-2-glycine into cultures of L5178YR cells. Cell inoculum at 0 hr, 5×10^5 viable cells/ml; antiserum dilution, 4×10^{-2}. △NRS, heat-inactivated normal rabbit serum; △n/1 I.S., batch of heat-inactivated immune rabbit serum.

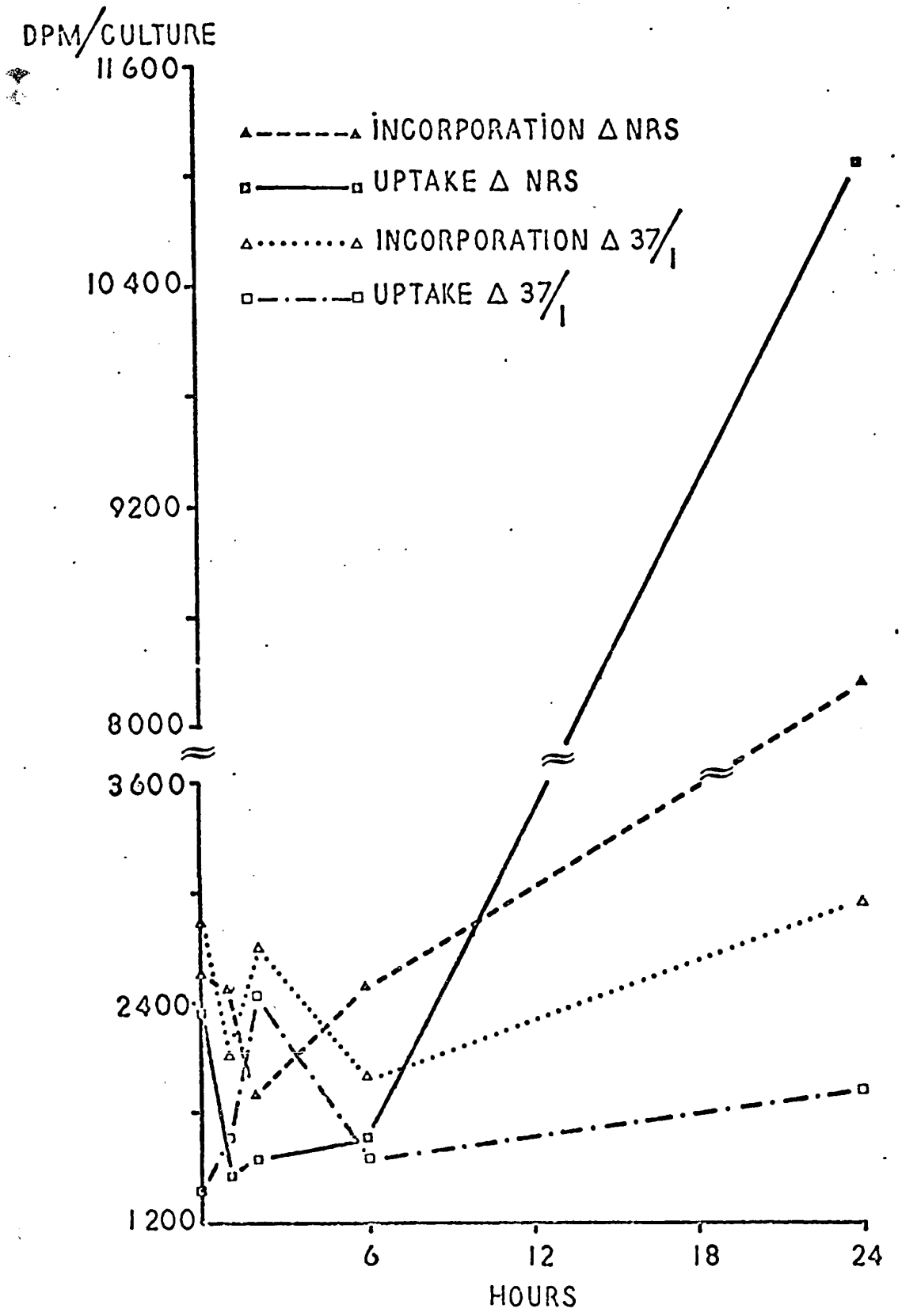
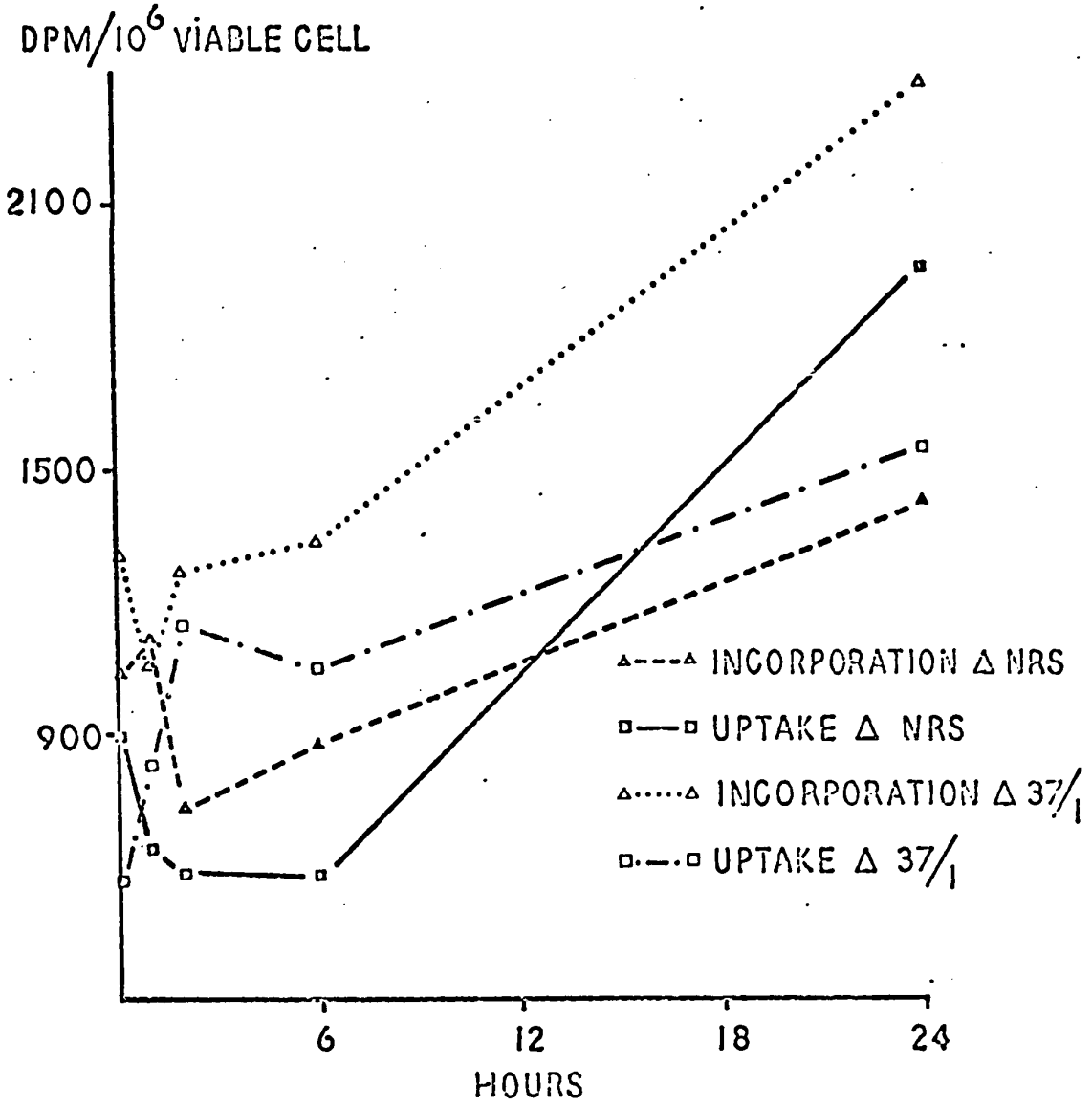

DPM/CULTURE
11 600
10 400
9200
8000
3600
2400
1 200
INCORPORATION Δ NRS
UPTAKE Δ NRS
INCORPORATION Δ 37/1
UPTAKE Δ 37/1
6
12
18
24
HOURS

Figure 4. ^{14}C-2-glycine uptake and incorporation/1 x 10^6 viable cells in cultures shown in Figure 3. ΔNRS, heat-inactivated normal rabbit serum; Δn/l I.S., batch of heat-inactivated immune rabbit serum.


DPM/10^6 VIABLE CELL
2100
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HOURS
INCORPORATION Δ NRS
UPTAKE Δ NRS
INCORPORATION Δ 37/1
UPTAKE Δ 37/1

Sodium succinate was able to partially spare the cells from the harmful effects of antibodies. Both the viable cell counts and the amino acid uptake and incorporation increased when 10 mM succinate was included in the system (Figure 5). Sodium malonate (10 mM) completely reversed the effects of succinate. Glucose and nicotinamide were found to be partially protective, but malate was without effect.

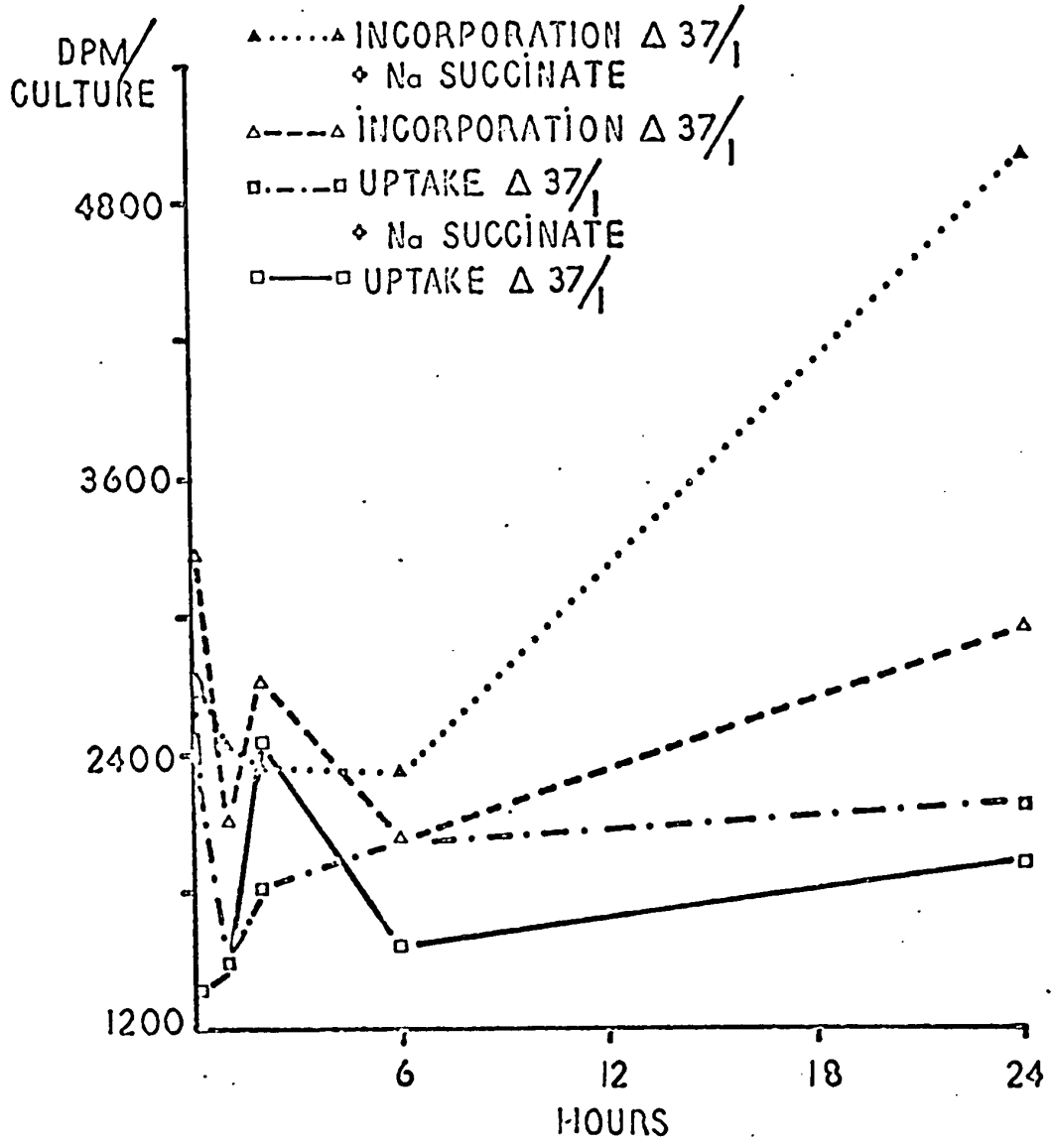
c. Studies on the Recovery of Antibody Treated Cells

At 24, 48, 72 and 96 hr after the initiation of the experiments, there was always a small population of surviving cells in which probably only multiplication was inhibited. They were larger than the usual cell size; bizarre-shaped cells were occasionally observed. The incorporation rate, but not the rate of uptake, of ^{14}C-2-glycine per 10^6 viable cells was much higher than that of the controls. Addition of succinate increased the incorporation rate still further. The proportion of very large cells was also increased. By replacing the medium which contained antisera with fresh antibody-free medium at 24 to 48 hr, the cells started to recover and to increase in number again.

4. Discussion

The present experiments demonstrate that heat-inactivated antiserum alone is able to inhibit the growth and to kill mouse leukemia L5178YR cells if the observation period is extended for longer than 6 hr. This finding suggests that the failure of some investigators to find any effect of heated antisera may have been due to the fact that they did not observe the cells for long enough.

Figure 5. Effects of 10 mM sodium succinate on the uptake and incorporation of ^{14}C-2-glycine into cultures of L5178YR cells affected by antiserum. Cell inoculum at 0 hr, 5×10^5 viable cells/ml; antiserum dilution, 4×10^{-2}.


DPM/
CULTURE
INCORPORATION Δ 37/1
Na SUCCINATE
INCORPORATION Δ 37/1
UPTAKE Δ 37/1
Na SUCCINATE
UPTAKE Δ 37/1
4800
3600
2400
1200
6
12
18
24
HOURS

As early as 1931, in fact Lumsden (1931) had noted that " in the absence of complement, antibodies cause agglutination of cells and not infrequently a precipitation reaction and gradual death are observed ". With experimental periods of at least 3 days, Habel et al. (1958) were able to show the inhibitory effects of anticellular antisera on cytopathogenic effects and on the yield of viruses in tissue culture, possibly through inhibition of cell metabolism. Kite et al. (1967) and Bartholomew, Kite and Rose (1967) have reported cytotoxic effects of anti-L cell and anti-HeLa cell antisera as shown by changes of microscopic morphology and medium pH within 5 days.

The slow killing action of antisera and the even slower decrease in total cell counts indicate that cell lysis is not the initial cause of cell death in the non-complement mediated system. Although all the sera agglutinated the cells, the agglutination titers of the sera were not parallel with the cytotoxic titers of the serum. The serum selected for metabolic studies (Δ37/1) was used in dilutions which exceeded the agglutination titer of this serum. Thus, clumping could not be contributing to the effects. The time of killing required by cytocidal agents, acting primarily through interference of biosynthetic mechanisms, seems to be related with the generation time and physiological state (e.g., different stage of cell cycle) of the cells.

Two hr after the addition of certain antisera, there was a transient increase both in viable cell counts and in uptake and incorporation of ^{14}C-2-glycine. The presence of noncycling tumor cells is

known (DeCosse & Gelfant, 1968) and long G2 leukemia cells in bovine leukemia have previously been observed (Yang et al., 1967). Whether or not the transient increase in viable cells noted in the present study is due to direct " stimulation " of noncycling G2 cells into mitosis by the antisera deserves further study. In a histological study it has been reported that in some instances antibodies could stimulate mitosis, suggesting that antibody-induced mitotic stimulation might in some cases, be an important factor in the tumor enhancement phenomenon (Gorer, 1962). DeCosse and Gelfant (1968) have recently reported the " release " of noncycling G2 mouse Ehrlich ELD ascites tumor cells into mitosis in vivo by antilymphocyte serum which they think acts by depressing the host immune mechanism.

The results with L5178YR cells (mouse leukemia lymphoblasts) are in sharp contrast with the stimulatory and transforming action of antisera on peripheral blood leukocytes which was first described by Gräsbeck, Nordman and De la Chapelle (1964). The response of the cells may depend on their differentiation level and be a result of the inherent variation in their thresholds to cell poisons. We have also observed such differences with phytohemagglutinin on L5178YR cells and peripheral blood leukocytes. Pauly, Caron and Suskind (1969) have reported that a sublethal dose of mercuric chloride caused optimal transformation of lymphocytes.

One can only speculate on the possible mechanisms for inhibition and killing by antisera, Some direct or indirect effect, triggered by the initial antigen-antibody reaction on the cell surface,

may be one of the causes although the possibility of an intracellular site of action for antibodies taken up by pinocytosis has not been completely excluded. In these experiments succinate, glucose and nicotinamide were all partially protective and the surviving cells were larger and could incorporate more amino acid into protein without a proportional increase in uptake. These observations suggest that general disturbances in the efficiency of substrate uptake and energy production from physical distortion of membrane-associated " enzyme systems " may be important. It has been reported that inhibition of energy production generally results in inhibition of cell division (Epel, 1963; Kihlman, 1966; Webster & Van't Hoff, 1969) and that the incorporation of amino acid into protein requires only 3 % in contrast to the uptake which may require as much as 50 % of the ATP utilized (Quastel, 1965). Furthermore, anti-D antibody has been reported to inhibit membrane-mediated ATP synthesis in membranes prepared from D-positive erythrocytes (Schrier, Moore & Ch'apella, 1968). Possible, indirect effects of the primary antigen-antibody reactions at the cell surface on intracytoplasmic changes could be implied from the work of Dumonde et al. (1961). Using a fluorescent antibody technique, they showed that antibodies were located exclusively on the cell surface although histochemical changes, such as marked activation of lysosomal acid phosphatase and dissociation of intracellular lipid-protein complex, were observed. Detailed study of the possible modes of action of the antibodies will be presented in Part IV.

The recovery studies included in this work showed that there

were cells which were more resistant to the harmful effects of antisera than was the cell population in general. The very large cells found (diameter 30.92±1.80u) in the surviving population probably represented polyploid cells. Immunoselection of polyploid forms in ascites tumors has been reported by Hauschka et al. (1956). Further studies on the resistant subpopulation will be presented in Part II.

5. Summary

An in vitro system employing rabbit serum medium-adapted subline of L5178Y cells and rabbit antisera was used.

Heat-inactivated antisera inhibited both uptake and incorporation of ^{14}C-2-glycine into cell protein and killed the cells. These effects became demonstrable only after 6 hr and reached a maximum after 24 to 48 hr. The decrease in total cell counts was even slower and the initial cause of cell death did not seem to result from cell lysis.

Sodium succinate partially protected the cells against killing or inhibition by antisera. Viable cell counts, and the uptake and incorporation of ^{14}C-2-glycine increased greatly. Malonate reversed completely the action of succinate. Apparently, the energy derived from the oxidation of succinate is sufficient for many cells to grow and to incorporate the amino acid into cell protein. Glucose and nicotinomide also showed similar protective effects.

Antiserum was observed to have a transient stimulatory effect on viable cell counts and the uptake and incorporation of ^{14}C-2-glycine

at about 2 hr after the addition of the antisera. Whether this is due to stimulation of noncycling G2 cells by antiserum is currently under study.

During the entire experimental period, i.e., up to 4 days, a small portion of the cell population remained viable. Many of these cells looked much larger and their incorporation rate, but not uptake rate, of ^{14}C-2-glycine per 10^6 viable cells was much higher than that of the controls. Addition of succinate increased the rate still further. Cultures treated with antiserum recovered if fresh medium without antisera was added at 24 to 48 hr.

Part II. Studies on the Resistant Subpopulation of Mouse Leukemia L5178YR Cells Treated with Antiserum: Inhibition of Colony Formation in Soft Agar by Antiserum and Comparison of the Responses of Parent Cells and Resistant Clones to Antiserum

1. Introduction

During studies with the growth inhibitory effects of anticellular antisera (in the absence of complement) on mouse leukemia L5178YR cells in culture, there was always a surviving subpopulation of cells even several days after the maximal inhibitory effects of antisera had been obtained. This suggested that the cell line is antigenically heterogeneous and contains a small fraction of cells which are immunologically resistant to antibodies directed to the majority of the cells. However, the possibility still existed that the surviving cells merely represented those which could modulate their antigenic expression in culture in the presence of antibodies (Old et al., 1968).

The following experiment was done to see if one of these hypotheses could explain the resistant clones. These clones were grown in soft agar. The technique had been modified by the addition of antiserum to see if agglutination by antibodies could be the primary cause of the inhibition of cell growth.

It was previously noted (Yang & Vas, 1970a) that agglutination titers did not correlate well with those of growth inhibition titers although Lindenmann(1964) had reported a close relationship

between them using tumor production in animals as her criterion.

2. Materials and Methods

L5178YR cells and the preparation of an antiserum against them has been previously described (Part I). The L5178YR cells used for these experiments had been through 186 to 209 passages.

a. Growth Inhibition in Soft Agar

The method used was a further modification of the technique reported by Macpherson and Montagnier (1964) and Eagle et al. (1970). Briefly, L5178YR cells at log phase of growth were counted and diluted with double strength rabbit serum-medium (20 % heat-inactivated, nomal rabbit serum in double strength Fischer's medium) to contain 2×10^5 viable cells per ml. Soft agar medium was prepared by mixing 1 % purified agar (50 C) (Difco Laboratories, Detroit, Michigan) in equal portions of glass-distilled water and double-strength Fischer's medium (37 C). A base layer of 7 ml was poured into 60 x 15 mm plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.), allowed to harden and then overlaid with 1.5 ml of the same medium but containing 1.5×10^5 viable cells. After the agar was solidified, 0.5 ml of dilutions of antisera in Fischer's medium was added evenly over the top of the agar. Antiserum dilutions were added on the top of the agar surface instead of being mixing with the cells in the soft agar to avoid cell agglutination at the higher serum concentrations. The plates were incubated in a humidified atmosphere of 5 % CO_2 in air at 37 C and examined daily under the inverted

microscope. Colonies of a diameter greater than 0.5 mm were counted after 7 to 14 days. Around two weeks some colonies had reached diameters of 0.8-2.0 mm. Occasionally colonies growing on the agar surface dissolved from too vigorous handling. However, no difficulty was found when the plates were carefully handled at the time of counting. An extra agar layer between the cell layer and the serum dilutions was not necessary. Five replicate plates were set up for each serum dilution unless otherwise stated. The average number of colonies was calculated.

b. Studies on Antiserum Resistant Clones

Resistant clones were picked up with Pasteur pipettes, recloned once and subcultured in Fischer's medium (10 % heated normal rabbit serum) for 4 passages before being tested for their ability tc form colonies in soft agar and to grow in Fischer's medium. Their ability to incorporate ^{3}H-5-uridine (Sp. Act. 1.0mc/0.0097mg) and ^{14}C-2-glycine (Sp. Act. 1.0mc/15.3mg) (New England Nuclear Corp., Boston, Mass.) into RNA and protein, respectively, was also tested. Using the same soft agar technique, the cells had to serve as their own control because cloning efficiencies differed slightly among cell suspensions even though they were of the same cell line. For the study of incorporation of ^{3}H-5-uridine and ^{14}C-2-glycine, Bollum's modified filter paper method was used. It is described in detail with the metabolic studies in Part IV. Three sets of triplicate tubes containing the 5 ml volume were inoculated to contain 2.5 x 10^{5} viable cells per ml and then either 0.01 or 0.05 ml antiserum was added.

The cultures were compared after 24 hr.

3. Results

Figure 6 shows the typical control Petri dish containing colonies of cells growing in soft agar after 8 day's incubation. Figure 7 shows the typical antiserum-treated Petri dish containing individual cells after the same incubation period. Antiserum dilutions of 1 in 20, 40, 80 and 160 in 0.5 ml of Fischer's medium added on the top of the cell containing agar layer inhibited colony formation by 98.2, 97.5, 91.4 and 39.1 %, respectively (Table 3). The test is very sensitive compared with other methods, especially when the diluting effect of both the cell containing agar layer (1.5 ml) and the basal agar layer (7.0 ml) are taken into consideration. By assuming an even diffusion of antiserum in both the cell containing agar layer and in the basal layer the final antiserum dilutions were 1/340, 1/680, 1/1360 and 1/2720, respectively.

Table 4 compares the inhibitory effects of various antisera (Δ39/1, Δ180/1 and Δ182/1). All were very inhibitory, even at a concentration as low as 1/80 (1/1360 final dilution).

Table 5 shows that, contrary to expectation, the cells cloned from the plates with 0.5 ml of a 1/20 dilution of antiserum (Δ180/1) and subpassaged in Fischer's medium did not show increased resistance over those of the original culture when tested with 0.5 ml of 1/5, 1/20 and 1/80 dilutions of the same antiserum. Table 6 further substantiates the above findings. Cells from the clones Fb and Fc after 4 further subpassages in Fischer's medium did not show an increase in resistance

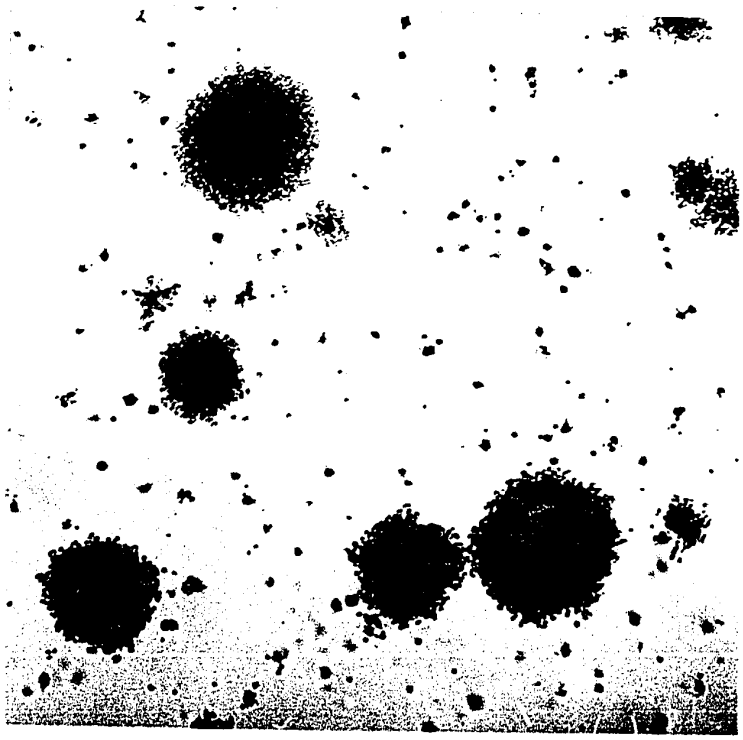


Figure 6 : Control Petri Dish Containing Colonies of Cells Growing in Soft Agar for 8 Days. 100X.

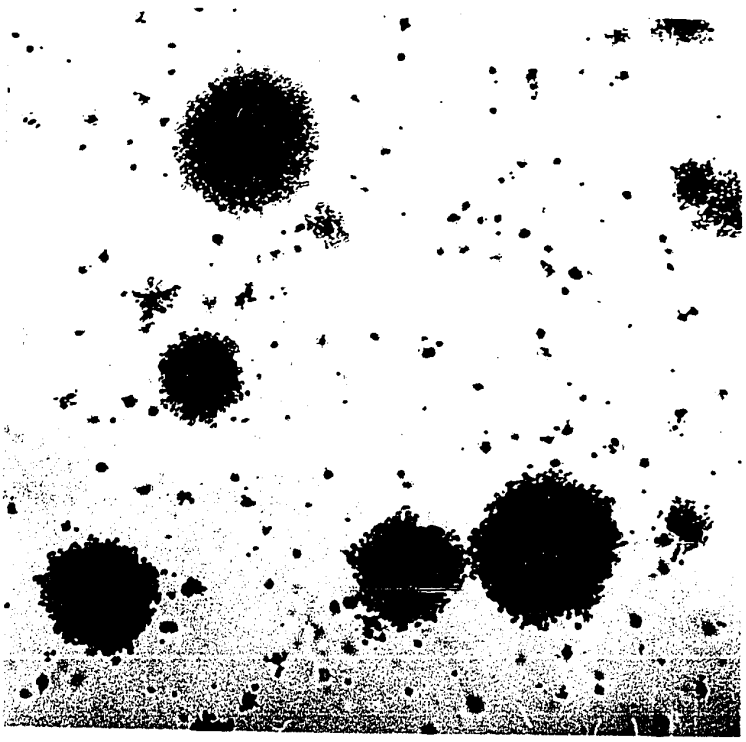


Figure 6 : Control Petri Dish Containing Colonies of Cells Growing in Soft Agar for 8 Days. 100X.

Figure 7 : Individual Cells in Soft Agar after 8 Days Incubation with Antiserum. 100X.

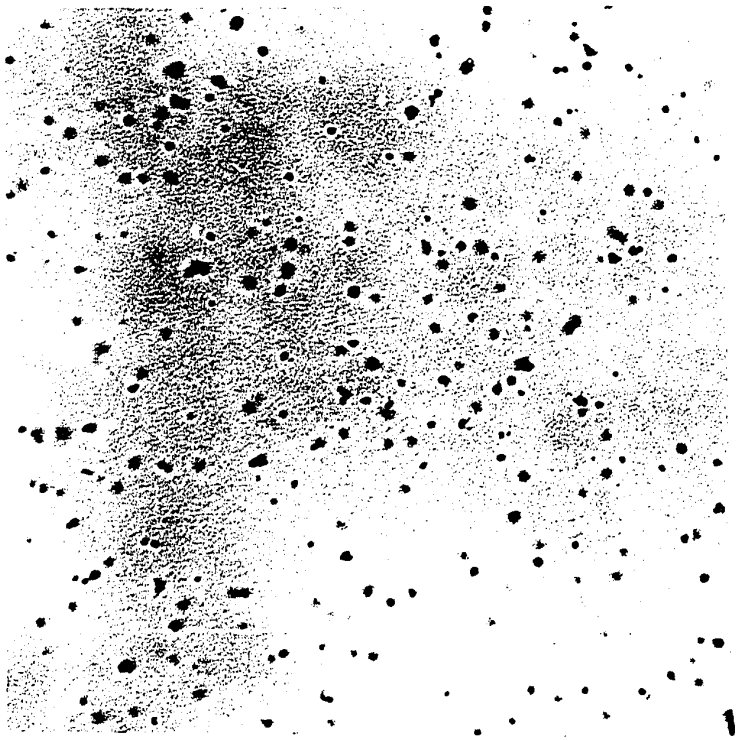


Figure 7 : Individual Cells in Soft Agar after 8 Days Incubation with Antiserum. 100X.

Table 3 : Colony Inhibition Test in Soft Agar with Serial Dilutions of Antiserum (Δ180/1)[a]

Serum dilutions[b]	Mean colony count ± SD[c]	% inhibition[d]
1 : 20	3.45 ± 2.40	98.2
1 : 40	5.00 ± 2.18	97.5
1 : 80	17.30 ± 4.47	91.4
1 : 160	123.1 ± 21.21	39.1
Control	202.4 ± 13.30	0.0

a: Total number of cells plated per dish: 1.5×10^5 viable cells. Colony counts made after 7 - 14 days.

b: 0.5 ml of 1:20, 1:40, 1:80 and 1:160 dilutions of antiserum.

c: Average of 4 - 5 plates ± Standard Deviation.

d: $\% \text{ inhibition} = \frac{\text{colony count (control) - colony count (antiserum)}}{\text{colony count (control)}} \times 100.$

Table 4 : Comparison of Inhibitory Effects of Individual Antisera on Colony Formation of L5178YR Cells in Soft Agar[a]

	Serum dilutions[b]			
Serum No.	1:20	1:40	1:80	1:160
Δ39/1	100.0[d]	96.0	63.0	NT[c]
Δ180/1	98.2	97.5	91.4	39.1
Δ182/1	100.0	91.0	86.0	NT

a,b,d: Same as Table 3.

c: Not tested.

Table 5 : Comparison of the Susceptibility of Parent L5178YR Cells and the Cells from Antiserum-resistant Clones[a]

Cells	Antiserum ($\Delta^{180}/1$) dilutions[b]			
	1:5	1:20	1:80	Control
L5178YR[c]	100.0[d]	96.0	63.0	0.0
A - 4[e]	90.0	80.0	63.0	0.0

a,b,d: Same as Table 3.

c: Parent cell population.

e: Cells from antiserum-resistant clones.

Table 6 : RNA and Protein Syntheses of Parent L5178YR Cells and the Cells from Antiserum-resistant Clones[a]

Serum dilutions[b]	Rates of ^{3}H-5-uridine incorporation			Rates of ^{14}C-2-glycine incorporation		
	L5178YR-201[c]	Fb-4	Fc-4	L5178YR-201	Fb-4	Fc-4
0	7492.2[d]	6549.7	5881.0	2120.6	1799.4	1543.9
1:500	5529.3	4703.0	4727.5	1860.2	1614.9	1617.2
1:100	3888.9	3310.4	3306.3	1373.4	1259.6	1206.6

a: Double labelling with 1 uc/ml each of ^{3}H-5-uridine and ^{14}C-2-glycine for 60 min; 24 hr cultures; initial cell inoculum, 2.5 x 10^{5} viable cells/ml.

b: Final antiserum (Δ180/1) dilutions in 5 ml culture.

c: L5178YR-201: parent cells at 201st passage. Fb-4 and Fc-4: cells from antiserum (Δ180/1)-resistant clones Fb and Fc, at 4th passage in liquid medium.

d: Average cpm of duplicate cultures x 1/25.

over that of the original cell population when tested in that medium in the presence of 0.01 and 0.05 ml of the antiserum (Δ180/1) i.e., at final concentrations of 1/500 and 1/100. RNA and protein syntheses were both inhibited to about the same magnititude in the antiserum doses tested (Table 6). Generally lower RNA and protein syntheses rates were found in both Fb and Fc cultures in either the presence or absence of the antiserum. This could be attributed to the lower growth potential of these cells in Fischer's medium (liquid).

4. Discussion

Anticellular antibodies, in the absence of complement, were found inhibitory to the colony formation of mouse leukemia L5178YR cells in soft agar. Application of antisera dilutions over the cell-containing agar layer, prevented agglutination of the cells by higher concentrations of antisera, substantiating a previous observation (Yang & Vas, 1970a) that agglutination titers did not parallel cytotoxic titers. It is still possible that membrane alterations could be the main cause of cell growth inhibition. The invaginations of cell membrane seen (Goldberg & Green, 1959; Easton, Goldberg and Green, 1962) at the ultrastructural level may reflect such changes; these experiments, however, covered only 30 to 60 min.

The present work favours the view that one of the major causes of cell inhibition is(see Part IV, also) some indirect mechanism triggered by the antigen-antibody reaction on the cell surface.

It was unexpected to find that the resistant clones did not show any appreciable increase in resistance to the same antiserum when

they were tested after 4 subpassages. Appearance of resistant clones in soft agar is reminiscent of the antigenic modulation phenomenon in mouse lymphoma cell reported by Old et al. (1968) and also recalls the cyclic variations in the antigenic expression of mouse lymphoma cells reported by Bjaring, Klein and Popp (1969). The possibility that sensitive revertants are selected during the subpassages cannot, however, be completely excluded.

In general, cell resistance may develop from either genotypic changes or phenotypic adaptation (in which the genotype of the cells does not change). In most cases, it is not easy to discover which of these mechanisms is responsible for the production of resistance.

Population of cell suspensions which have survived antiserum treatment contain increased numbers of very large cells which may be polyploid. Attempts to clone these cells may prove successful when more cells have been examined. It was interesting to note that very large cells develop again spontaneously within 4 further passages of the ordinary clones in Fischer's medium. This suggests that the increase in the number of these cells was due to the selection pressure afforded by antiserum.

5. Summary

Heat-inactivated antiserum, in the absence of complement, inhibits colony formation of mouse leukemia L5178YR cells in soft agar. Cells derived from resistant clones in these plates showed the same sensitivity to antiserum as the parent population. Agglutination of the cells is prevented by placing the antiserum

dilutions over the cell-containing agar layer. This clearly indicates that cell agglutination by antiserum is not necessarily the essential cause of growth inhibition.

Part III. Effect of Cyclic 3',5'-Adenosine Monophosphate on Mouse Leukemia L5178YR Cells in Culture[3]

1. Introduction

The importance of adenosine 3',5'-monophosphate (cyclic AMP) to the normal cell function and metabolism of many tissues and organs is becoming increasingly apparent (Robison, Butcher & Sutherland, 1968; Sutherland, 1970). Cyclic AMP has been recently reported to inhibit the growth of transplanted NKL-lymphosarcoma in mice and the multiplication, as measured by viable counts, of tumorigenic cell lines in vitro (Ryan & Heidrick, 1968; Gericke & Chandra, 1969; Heidrick & Ryan, 1970). However, the mechanism by which cyclic AMP inhibits the cancer cells is, at present, unknown.

Results of the study of the inhibitory action of heat-inactivated antisera, in the absence of complement, on mouse leukemia L5178YR cell cultures caused speculation that some indirect mechanisms triggered by antibody-antigen reactions on the cell membrane might be involved (Yang & Vas, 1970b). The growth inhibitory effects of the antisera on the cells occur rather slowly, as do the hormonal effects. Both start after 4 to 6 hr and have maximal effects at 24 to 48 hr. Systems " around the pyruvic acid cycle " seem to be involved (Ide, 1969). As has been mentioned previously, glucose, succinate and nicotinamide, but not malate, were able to alleviate, at least partially the harmful effects of the antisera. This phenomenon is reminiscent

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(3) The result has been in the press in Experientia, 1971.

of the second messenger effect found in hormonal systems. Besides, the activation of adenyl cyclase in the sea urchin egg membranes at fertilization has recently been reported (Castaneda & Tyler, 1968). The following investigation was undertaken to find if cyclic AMP could mimic the effects of antisera on L5178YR cells.

2. Materials and Methods

The experimental method was a combination of that previously described (Part I) and modified Bollum's method (Bollum, 1959; Byfield & Scharbaum, 1966). Two sets of series of triplicate Bellco tissue culture tubes received an inoculum of 1×10^5 or 2.5×10^5 viable cells per ml in 5 ml amounts of medium. The smaller inoculum was used for viable cell counts only, while the larger was used for both viable counts and radioisotope labeling studies. Adenosine 3',5'-monophosphate (cyclic AMP, Calbiochem, Los Angels, Calif.) and N^6-2'-O-dibutyryl-adenosine 3',5'-monophosphate (dibutyryl cyclic AMP, Boehringer-Mannheim, New York) were dissolved in warm, distilled water to make stock solutions of 10 mg per ml and sterilized by passage through a washed Millipore filter. Similar volumes of filtered distilled water were used as controls. For the dose-response study, 0.2 ml of two-fold dilutions of the stock solutions were added to the cultures at the start of the experiments to make final concentrations of 400 ug, 200 ug, 100 ug, 50 ug, 25 ug and 12.5 ug per ml of culture. At 24 and 48 hr, viable counts were made. For the time-course study, a final concentration of 200 ug/ml (0.40 mM for dibutyryl cyclic AMP and 0.53 mM for cyclic AMP) was used.

At 0, 1, 2, 4, 6, 8, 24 and 48 hr after the start of the experiment, each set of triplicate cultures was pooled, mixed, redistributed equally into 3 tubes and ^{3}H-thymidine (Sp. Act. 10mc/0.036mg), ^{3}H-uridine (Sp. Act. 1.0mc/0.035mg), and ^{14}C-2-glycine (Sp. Act. 0.05mc/0.9mg) (New England Nuclear Corp., Boston, Mass.) were added to make final concentrations of 1 uc, 4 uc and 1 uc per ml, respectively. These were then pulse labeled for 60 min in 37 C water bath. Further incorporation was stopped by chilling. Viable cell counts were made and the cells were processed by conventional TCA extraction methods (see Part I) and modified Bollum's filter paper method (Byfield & Scharbaum, 1966), and finally assayed in the liquid scintillation spectrometer. The experiments were repeated 2 to 4 times.

3. Results and Discussion

Table 7 shows the results of the dose-response study. Dibutyryl cyclic AMP inhibited cell growth, even at the lowest concentration used, i.e., 12.5 ug per ml (0.025 mM), while cyclic AMP was much less effective and inhibited the cells only at the concentration of 200 ug per ml (0.53 mM) or higher. At the concentrations of 50-25 ug per ml (0.133-0.066 mM), cyclic AMP showed some stimulatory effects. These are in accord with the known facts that dibutyryl cyclic AMP penetrates the cells better and is more resistant to the action of specific phosphodiesterase (Posternak, Sutherland & Henion, 1962; Butcher et al., 1965). Furthermore, it shows that the increased susceptibility to cyclic AMP, in contrast to dibutyryl cyclic AMP, is

Table 7 : Comparison of Inhibition of L5178YR Cells by Dibutyryl Cyclic AMP and Cyclic AMP

Compound	Concentration (ug/ml)	Concentration (mM)	Inhibition (%)[a] 24 hr	Inhibition (%)[a] 48 hr
Dibutyryl cyclic AMP	400	0.800	59	94
	200	0.400	60	85
	100	0.200	67	71
Cyclic AMP	400	1.060	43	73
	200	0.530	-10	22
	100	0.265	8	1

a: $\%\ \text{inhibition} = \frac{\text{viable cell count (control)} - \text{viable cell count (cAMP)}}{\text{viable cell count (control)}} \times 100$

not necessarily the property of tumorigenic cell lines as has been reported by others (Heidrick & Ryan, 1970). Figure 8 shows that the rate of RNA synthesis decreased significantly after 4 to 6 hr, while the decrease in protein synthesis was not demonstrable until after 8 hr (Figure 9). Marked differences in the rate of DNA synthesis also occurred only after 8 hr. In repeated trials there was a consistent slight decrease in the rate of DNA synthesis from the initiation of the experiments onward. Such results suggest that the cells entering into the DNA synthesizing S phase of the cell cycle are preferentially inhibited, and the rates of DNA synthesis expressed in terms of 10^6 viable cells clearly confirm that this is the case. Under the experimental conditions used, the cells showed a semi-synchronous wave at 8 hr. Selective inhibition at the site or stage of the initiation of DNA replication and RNA transcription seems to be the earliest biosynthetic mechanism affected by cyclic AMP, substantiating the findings of Langan (1969) and Perlman, De Crombrugghe and Pastan (1969). Langan shows that in vivo histones serve as a substrate for the cyclic AMP-dependent protein kinase. He postulates that increased histone phosphorylation brought about by hormone administration may provide a mechanism for the induction of RNA and protein synthesis in target tissues. Perlman et al. (1969) have shown that stimulation of β-galactosidase synthesis by cyclic AMP occurs at the level of transcription of RNA and that the lac operon promotor is the site of action of the cyclic AMP. Whether the response of a cell stimulus is stimulatory or inhibitory may

Figure 8 : Effects of dibutyryl cyclic AMP on the rates of DNA and RNA syntheses in cultures of L5178YR cells. Cell inoculum at 0 hr, 2.5×10^5 viable cells/ml; dibutyryl cyclic AMP conc., 200 ug/ml (0.4 mM). Labeling time, 60 min.

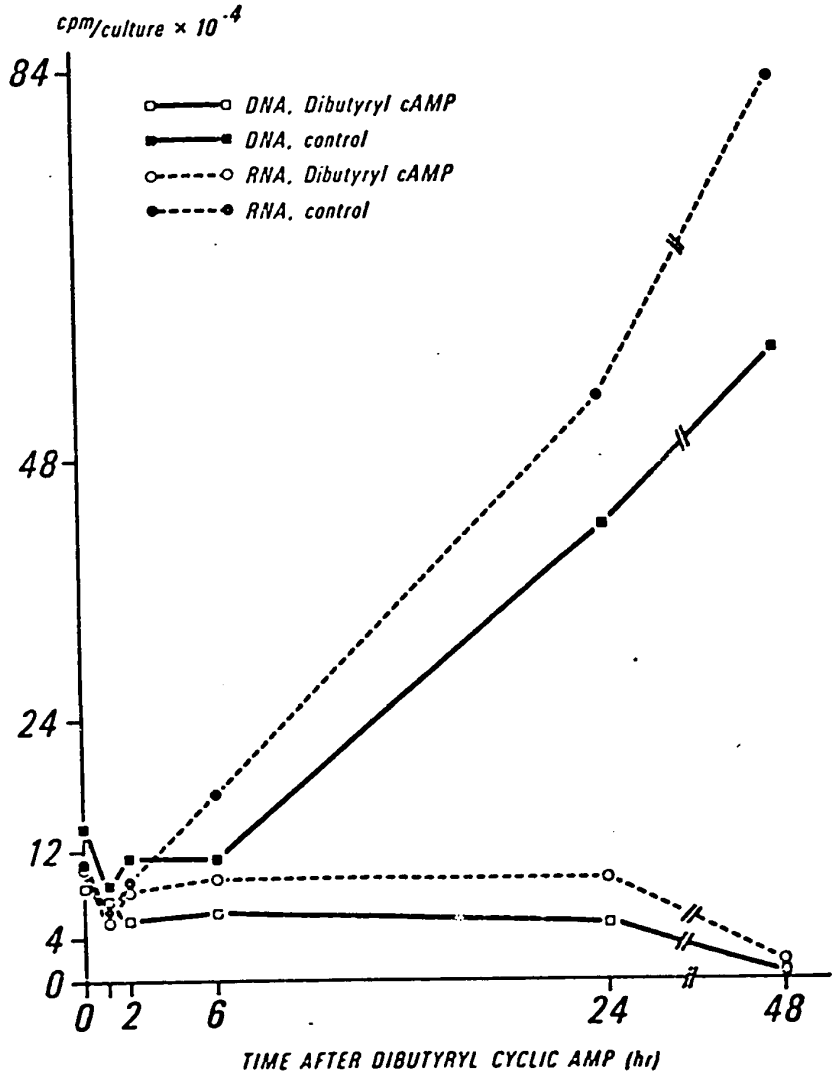
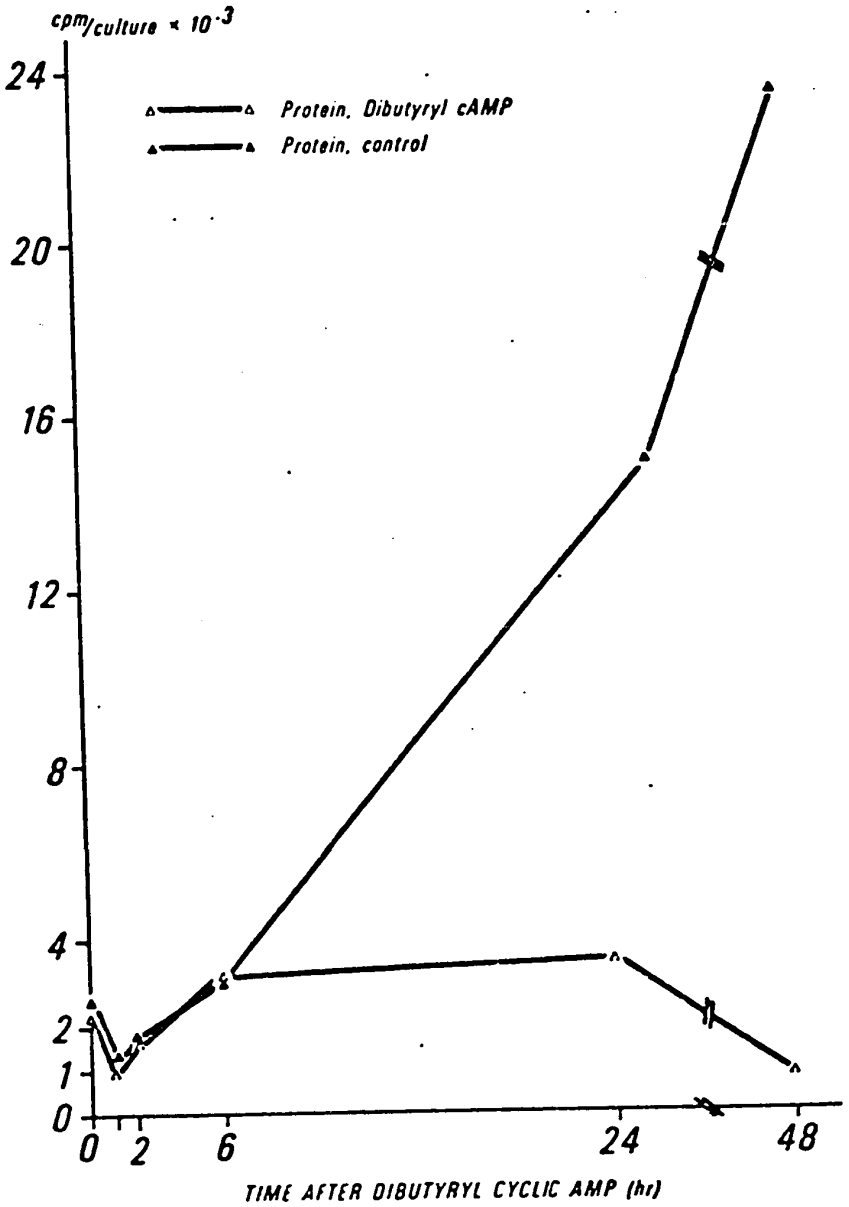

cpm/culture × 10⁻⁴
84
48
24
12
4
0
DNA, Dibutyryl cAMP
DNA, control
RNA, Dibutyryl cAMP
RNA, control
0
2
6
24
48
TIME AFTER DIBUTYRYL CYCLIC AMP (hr)

Figure 9 : Effects of dibutyryl cyclic AMP on the rate of protein synthesis in cultures of L5178YR cell. Experimental conditions are the same as in Fig. 8.


cpm/culture × 10⁻³
24
20
16
12
8
4
2
1
0
Protein, Dibutyryl cAMP
Protein, control
0
2
6
24
48
TIME AFTER DIBUTYRYL CYCLIC AMP (hr)

depend on the physiological and differential state of the cells. This has been shown for several other systems, e.g., in the responses of lymphocytes and lymphoblasts towards anticellular antibodies (Gräsbeck et al., 1964; Yang & Vas, 1970a), phytohemagglutinin (Nowell, 1960; Yang et al., 1967), and cyclic AMP (MacManus & Whitfield, 1969; Yang & Vas, 1970b). Finally, the decrease in the rate of ^{3}H-thymidine incorporation found in the cyclic AMP treated cells is unlikely to be caused by the activation of thymidine phosphorylase which breaks down thymidine to thymine. Rabinowitz and Wilhite (1969) have shown recently that cyclic AMP did not alter the enzymes in the thymidine salvage pathway in either normal or leukemia leukocytes.

4. Summary

Cyclic AMP inhibits the growth of mouse leukemia L5178YR cells in culture. The rates of RNA and protein synthesis decrease significantly after 4 to 6 hr, and 8 hr respectively. The rate of DNA synthesis decreases slightly within 1 to 6 hr, and markedly after 8 hr.

Part IV. Possible Mediation of the Inhibitory Effects of Antiserum on Mouse Leukemia L5178YR Cells by Cyclic 3',5'-Adenosine Monophosphate[4]

1. Introduction

During the course of studies on the growth inhibitory effects of anticellular antibodies on mouse leukemia L5178YR cells in culture (see Part I), it was speculated that major causes might be some indirect mechanisms triggered as a result of antigen-antibody reactions on the cell membrane. Inhibitory effects of the antisera on growth are a rather slow process (see Part I) as are hormonal effects on the cells (Trowell, 1965). As stated previously, they start 6 hr after the addition of antisera to the cells, and have their maximal effects after 24 to 48 hr. Systems " around the pyruvic acid cycle " seem to be involved (Ide, 1969). For instance, glucose (Makman & Sutherland, 1965), succinate and nicotinamide (Krishna et al., 1966) but not malate (Sanwal & Smando, 1968), were able to alleviate, at least partly, the harmful effects of antisera (Yang & Vas, 1970a). This phenomenon is similar to the second messenger mechanism found in hormonal systems (Robison et al., 1968; Sutherland, 1970). Since then, activation of adenyl cyclase in the sea urchin egg membranes was also reported to occur at the time of fertilization (Castaneda & Tyler, 1968),

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(4) Part of the results has been published in Proc. Canadian Fed. Biol. Soc. 13:509, 1970.

and therefore the effects of cyclic AMP on the cells in culture were tested (see Part III).

In the present set of experiments, the time-course effects of cyclic AMP and antibodies on the biosynthetic mechanisms of L5178YR cells in culture were investigated. Moreover, studies were made to see whether theophylline, which is a specific inhibitor of phosphodiesterase, can potentiate the effects of antibodies, and if antibodies can stimulate an increase in cyclic AMP levels.

2. Materials and Methods

a. L5178YR Cells

These have been previously described in Part I.

b. Antisera

The sera were prepared as before, collected aseptically and stored at -70 C. They were heat-inactivated (56 C, 30 min) just before use. Heat-inactivated pre-immune sera and other normal sera were used as controls.

c. Metabolic Studies

For the time-course study of the effects of antiserum and cyclic AMP, two sets of triplicate cultures received an inoculum of 1×10^5 viable cells per ml (for viable counts) or 2.5×10^5 per ml (for both the viable counts and the radio-isotope labeling experiments). The cultures were again set up in 5 ml amounts. Antisera (0.2 ml) or dibutyryl cyclic AMP (0.2 ml of 0.5 % solution; Boehringer-Mannheim, New York) was added to the

cultures at the start of the experiments. Experiments of 8 hr duration or less were done in a 37 C water bath with the tubes in an upright position. For experiments longer than 8 hr the tubes were placed into an incubator in a slanted position, again to obtain maximal air space in the tube. At 0, 1, 2, 4, 6, 8, 24 and 48 hr after the start of the experiments, triplicate cultures from each set were pooled, mixed, redistributed equally into 3 tubes and ^{3}H-thymidine (Sp. Act. 1.0mc/0.036mg), ^{3}H-uridine (Sp. Act. 1.0mc/0.035mg-0.082mg) (or ^{3}H-5-uridine, Sp. Act. 1.0mc/0.0097mg) and ^{14}C-2-glycine (Sp. Act. 1.0mc/15.3mg) (New England Nuclear Corp., Boston, Mass.) were added to make final concentrations of 1 uc, 4 uc, and 1 uc per ml, respectively. They were then pulse-labeled for 60 min in a 37 C water bath. Incorporation was stopped by chilling, viable cell counts were made and the cells processed by both the conventional TCA extraction method for nucleic acid, and protein (see Part I) and the modified Bollum's filter paper method (Bollum, 1959; Byfield & Scharbaun, 1966). For modified Bollum's method, 4 samples of 0.1 ml of each of the cell suspensions were removed by an automatic Eppendorf pipette (Eppendorf Geratebau, Netheler and Hinz GMBH, Hamburg) and placed onto a 1 inch Whatman 3 MM filter paper disc. The wet saturated filter paper disc was transferred into ice-cold TCA in Erhlenmeyer flasks (5 % TCA was used for nucleic acid and 10 %, for protein). At the end of the experiment, the accumulated discs were washed by gently swirling the flasks. After three washes of 15 min each with 5 ml of 5 % TCA per disc they were then washed twice each both in 95 % ethanol-ether (3:1)

and in ether in order to remove water and TCA. The discs were spread out on filter paper and allowed to air dry. Bray's scintillation fluid was used for the extracted material but toluene scintillation fluid (0.6 % 2,5-diphenyloxazole and 0.01 % of p-bis-2-(5-phenyloxazole) benzene in toluene) was used for the discs. Finally both were assayed in the spectrometer. For the other experiments, such as those on the effects of theophylline and on the heat stability of antisera, a double labeling method was employed. It used ^{3}H-5-uridine and ^{14}C-2-glycine in amounts to give a final concentration of 1 uc each per ml. Sets of duplicate series of cultures containing 2.5×10^{5} viable cells per ml were set up and after 24 hr of incubation, pulse labeled with the precursors for 60 min in the water bath. The cultures were then processed by Bollum's method.

d. Nature of the Antibody

i. Relative Heat Stability of Antibodies

The inhibitory activity of the antisera was tested after heating it at 65 C for 60 and for 90 min and comparing these samples with one that was inactivated at 56 C for 30 min. Duplicate (5 ml) cultures containing 2.5×10^{5} viable cells per ml were set up and 0, 0.05, 0.2 and 0.4 ml of the antiserum preparations added. The inhibitory effects were compared after 24 hr using the metabolic method described in the previous section.

ii. Specificity of Antibodies

The method for antibody recovery from erythrocytes by heat was originally developed by Landsteiner and

Miller but later modified by Rosenfield and Kochwa (1967). Further modification was made to elute antibodies which had been absorbed to the cells in the present experiment. One volume (0.6 ml) of washed and packed L5178YR cells was applied to 3 volumes (1.8 ml) of antiserum (Δ36/1) and incubated at 37 C for 1 hr. The antibody-sensitized cells were then washed at least three times, each time with at least 10 volumes of saline, and then suspended in 3 volumes (1.8 ml) of Fischer's medium and heated at 56 C for 20 min. After centrifugation at 56 C to sediment the cells the supernatant, containing the eluate, was removed immediately so that no antibodies would reabsorb. The supernatant was tested as described above for its antibody activity.

e. Potentiation of the Inhibitory Effects of Antibody by Theophylline

Since preliminary studies have shown that theophylline at high concentrations was toxic by itself, the box titration method was used with the combinations of 0, 1/500, 1/100 antiserum (Δ180/1) dilutions and 0, 0.1, 0.25, 0.5 and 1.0 mM theophylline (Calbiochem, Los Angels, Calif.). The effect of theophylline on the cells treated with the antiserum was measured after 24 hr using the metabolic study method mentioned previously.

f. Measurement of Cyclic AMP Accumulation

The assay method developed by Humes, Roundbehler and Kuehl (1969) for measuring adenyl cyclase activity in intact cells and the radioimmunoassay method for the measurement

of cyclic AMP developed by Steiner et al. (1969) were used.

i. Adenyl Cyclase Activity in Intact Cells

L5178YR cells (2-3 liters) were centrifuged, resuspended in 20-30 ml of Fischer's medium at a concentration of 2.0 to 2.5 x 10^7 per ml and incubated for 90 min at 37 C in a shaking water bath with ^{14}C-8-adenine (Sp. Act. 50.0mc/m mole) or ^{14}C-8-adenosine (Sp. Act. 55 mc/m mole) (Schwarz BioResearch, Inc., Orangeburg, N. Y.) in a final concentration of 1 uc per ml. The cells were then distributed in 1 ml aliquots in duplicate series of tubes, and 0.2 ml of antiserum or normal control serum, and 0.1 ml of a 50 mM solution of theophylline were added to make a final concentration of 5 mM (Streeto & Reddy, 1967). The cultures were incubated and after 0, 2, 5, 10, 40 and 60 min, the enzymatic reactions were terminated by the addition of 0.2 ml of 0.6 M TCA. Then, 0.05 ml of an aqueous solution containing 0.01 M each of ATP, ADP, 5'AMP, cyclic AMP, adenosine and adenine (Calbiochem, Los Angels, Calif.) was added to facilitate the recovery of the labeled nucleotides. In addition, ^{3}H-8-adenosine-3',5'-cyclic phosphate (^{3}H-cyclic AMP, Sp. Act. 16.3c/m mole; Schwarz BioResearch, Inc., Orangeburg, N. Y.) 0.2 uc was added for co-chromatography. The cells ruptured from such treatment. The insoluble material was removed by centrifugation for 5 min at 1000 x g. The resulting pellet was washed with 0.2 ml of 2 % TCA, centrifuged and the supernatant was taken and added to the initial TCA extract. The combined supernatants were passed through an ion exchange resin (AG50W-X2(H^+),

100-200 mesh (0.7 x 7 cm), Bio-Rad Laboratories, Richmond, Calif.), and then eluted with water. The initial 4 ml eluant was discarded and the cyclic AMP was collected in the subsequent 10 ml fraction. After treatment with 0.5 ml each of aqueous 8 % zinc sulfate and 0.3 N barium hydroxide, the slurry was centrifuged for 5 min at 1000 x g. The resulting supernatant was again subjected to this precipitation procedure and then lyophilized. The residue (according to Humes et al. (1969) containing mostly ^{14}C-cyclic AMP) was dissolved in 0.3 ml of water and the radioactivity of a 0.1 ml aliquot was measured in toluene scintillation fluid after being soaked and dried on 1 inch^2 Whatman 3 MM filter paper. Another 0.03 ml aliquot was applied to Whatman 3 MM paper by three successive applications of 0.01 ml each and then developed for 16 hr in a solvent consisting of 1 M ammonium acetate (pH 7.5) and ethanol (30:75). This paper chromatography system allowed good separation of ATP, ADP, 5'AMP, adenine and adenosine from cyclic AMP. For good resolution this step was needed in addition to the above described AG50W-X2(H^+) and zinc sulfate-barium hydroxide procedure (Krishna, Weiss & Brodie, 1968; Krishna & Birnbaumer, 1970). The strips were examined by ultraviolet light and spots on the chromatogram corresponding to co-chromatographed, authentic cyclic AMP were cut out and placed in toluene scintillation fluid. Double isotope scintillation counting was done directly on the paper sections. All ^{14}C counts were corrected to 100 % on the basis of the tritium recovered.

ii. Radio-immunoassay of Cyclic AMP

The radio-immunoassay system developed by Steiner et al. (1969) is based upon the competition of cyclic AMP with a labeled cyclic AMP derivative. The derivative has a high specific activity for binding sites on the antibody specific for the cyclic necleotide. Rabbit anti-cyclic AMP antiserum (produced by immunizing rabbits with 2'O-succinyl cyclic AMP-human serum albumin) and ^{125}I-succinyl cyclic AMP-tyrosine methyl ester antigen (^{125}I-SCAMP-TME) were purchased from Collaborative Res., Inc., Waltham, Mass. Goat anti-rabbit serum was purchased from Hyland Laboratories, Los Angels, Calif.

1). Preparation of Tissue Extracts

L5178YR cells treated with antiserum as described above were added to 0.5 ml of 6 % TCA, and freeze-thawed several times. After centrifugation at 4000 rpm for 15 min, the supernatant was removed and extracted three times with 5 ml of water-saturated petroleum ether. The extracted aqueous phase was heated at 70-80 C for 2 min, lyophilized, and the dry residue dissolved in 0.3 ml of 0.05 M sodium acetate buffer (pH 6.2). This extract was used directly in the immunoassay system.

2). The Immunoassay Procedure

Anti-succinyl cyclic AMP rabbit antibody (globulin fraction) was diluted 1:2000, a dilution at which 30-40 % of the ^{125}I-SCAMP-TME was precipitated in the presence of excess goat anti-rabbit IgG. One-tenth ml of the rabbit antibody

was added to 0.3 ml of either tissue extract or sodium acetate buffer; the buffer contained non-radioactive cyclic AMP (0.38, 0.75, 1.5, 3.0, 6.0, 12.0, 24.0, 47.0, 94.0, 188.0, 375.0, 750.0, 1500.0 picomoles per 0.3 ml). One-tenth ml of a solution of ^{125}I-SCAMP-TME, diluted to contain approximately 4000-8000 cpm (0.05-0.1 picomoles), was then added to bring the reaction volume to 0.5 ml and the tubes were incubated for 3 hr at 4 C. Excess goat anti-rabbit IgG serum was added and the tubes were subsequently incubated overnight in the cold. The tubes were then added with 2.0 ml of the buffer and centrifuged at 4000 rpm at 4 C for 30 min, and the precipitate dissolved in 0.5 ml of Hyamin before being counted in a gamma spectrometer. All analyses were carried out in either duplicate or triplicate.

3. Results

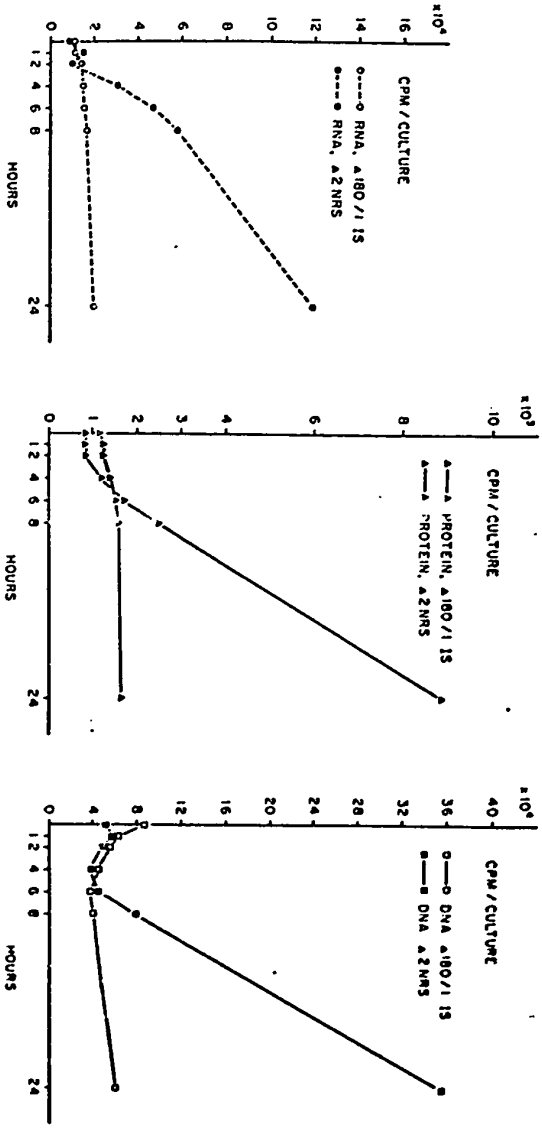
a. Comparison of the Effects of Antiserum and Cyclic AMP on DNA, RNA and Protein Syntheses

As shown in Figure 10, the rate of RNA synthesis decreased significantly at 4 hr in the cultures treated with antiserum, while the decrease in protein synthesis was not demonstrable until 6 to 8 hr (Figure 11). Differences in the rate of DNA synthesis occurred only after 8 hr (Figure 12). The sequence of metabolic events in the cultures treated with antiserum is remarkably similar to those treated with cyclic AMP. The sequence of metabolic events in the cultures treated with cyclic AMP is essentially similar to that presented in Figures 8 and 9 in

Figure 10 : Time effects of antiserum (Δ180/1) on the rate of RNA synthesis in cultures of L5178YR cells. Cell inoculum at 0 hr, 2.5×10^5 viable cells/ml; antiserum conc. 0.2ml/5 ml (i.e., 4×10^{-2}). Labeling time, 60 min.

Figure 11 : Time effects of antiserum (Δ180/1) on the rate of protein synthesis in cultures of L5178YR cells. Experimental conditions are the same as in Fig. 10.

Figure 12 : Time effects of antiserum (Δ180/1) on the rate of DNA synthesis in cultures of L5178YR cells. Experimental conditions are the same as in Fig. 10


CPM / CULTURE
o---o RNA, ▲180 / I IS
●---● RNA, ▲2 NRS
HOURS
CPM / CULTURE
▲—▲ PROTEIN, ▲180 / I IS
▲—▲ PROTEIN, ▲2 NRS
HOURS
CPM / CULTURE
o—o DNA ▲180 / I IS
■—■ DNA ▲2 NRS
HOURS

Part III. Thus cyclic AMP mimics many of the major effects of antiserum on the cells.

b. Nature of Antibody

i. Relative Heat Stability of Antibodies

Since the antisera could inhibit the growth of the cells at rather high titers (i.e., 1/500 or higher in the absence of complement), it was decided to find out whether the IgG class of immunoglobulins was alone responsible or if the IgM was also implicated. A heat inactivation method was used because it has been suggested that inactivation at 65 C for 1 hr is a more reliable measure of macroglobulin antibody than is 2-mercaptoethanol treatment (Locke & Segre, 1965; Pike, 1967). Furthermore, preliminary studies have indicated that 2-mercaptoethanol at concentrations lower than the conventionably used 0.1 M (Schur & Christian, 1964) was still too toxic for the cells.

As shown in Table 8, heating the antisera at 65 C for either 60 min or 90 min lowers the titers only slightly more than those of the antisera inactivated at 56 C for 30 min. The rates of both RNA and protein syntheses were very slightly affected at the antiserum concentrations studied (1/100, 1/25 and 1/12.5). Based on the reports of others on the heat stability of immunoglobulins (Locke & Segre, 1965; Pike, 1967), it seems most likely that the predominant type of antibody globulin is of the IgG class.

ii. Specificity of Antibodies

Antibodies present in the antiserum

Table 8 : Comparison of the Inhibitory Activity of Antiserum (Δ36/1), Heated at 56 C for 30 min and at 65 C for 60 and 90 min, on the Rates of RNA and Protein Syntheses in Cultures of L5178YR Cells[a]

Serum dilution[b]	Rates of ^{3}H-5-uridine incorporation			Rates of ^{14}C-2-glycine incorporation		
	56C 30'	65C 60'	65C 90'	56C 30'	65C 60'	65C 90'
0	5235 ± 532[c]			2141 ± 66		
1:100	2050±69	2528±206	2424±206	1004±30	1288±95	1253±102
	(60%)[d]	(51%)	(53%)	(53%)	(39%)	(41%)
1:25	1656±194	1851±192	1881±150	664±18	793±65	811±75
	(68%)	(64%)	(64%)	(68%)	(62%)	(62%)
1:12.5	1423±146	1638±158	1729±150	531±23	546±50	635±90
	(72%)	(68%)	(66%)	(75%)	(74%)	(70%)

a, b: Same as Table 6.

c: (Mean cpm ± S.D.) x 1/25. Average of 3 cultures.

d: $$\% \text{ inhibition} = \frac{\text{cpm (control)} - \text{cpm (antiserum)}}{\text{cpm (control)}} \times 100.$$

presumably account for its inhibitory effect on cell growth. The antiserum could be absorbed by the cells and later eluted. The antibodies eluted from the sensitized L5178YR cells retained their specific activities at quite high levels as is shown by their inhibition of both RNA and protein syntheses (Table 9).

c. Potentiation of the Inhibitory Effects of Antiserum by Theophylline

As shown in Figure 13 theophylline, at a concentration of 0.25 mM, potentiates the effect of a 1/100 dilution of antiserum (Δ179/1) when measured by the incorporation of ^{3}H-5-uridine into RNA. This suggests that antibodies and theophylline have synergistic effects through the common mediator, cyclic AMP.

d. Measurement of Cyclic AMP Accumulation

As shown in Figure 14, a definite increase in the de novo synthesis of cyclic AMP occurs 2 min after stimulation of the cells by the antibody. The level persisted for the 40 to 60 min that the cultures were studied, substantiating the hypothesis that the increase in the level of cyclic AMP was one of the major initial changes after the antigen-antibody reactions on the cell membrane.

4. Discussion

This experiment has shown that antiserum affects the cell metabolism in a sequential order. The rate of RNA synthesis decreased significantly by 4 hr while the decrease in DNA and protein syntheses was not demonstrable until after 6 to 8 hr

Table 9 : Inhibitory Activity of Antibodies Eluted from Sensitized L5178YR Cells[a]

Serum dilution[b]	Rates of ^{3}H-5-uridine incorporation		Rates of ^{14}C-2-glycine incorporation	
	56C 30'	Eluate[e]	56C 30'	Eluate
0	11016.8		1645.5	
1:100	5417.0[c]	7890.1	1122.1	1034.7
	(50%)[d]	(28%)	(31%)	(37%)
1:25	3241.6	6122.3	455.2	726.7
	(70%)	(44%)	(72%)	(55%)
1:12.5	2952.2	4715.9	324.0	602.0
	(73%)	(57%)	(80%)	(63%)

a,b: Same as Table 6.

c: Average cpm of duplicate cultures x 1/25.

d: Same as Table 8.

e: Eluate reconstituted to original serum volume.

Figure 13 : Effects of theophylline and antiserum (179/1) on the rate of RNA synthesis in cultures of L5178YR cells. Cell inoculum at 0 hr, 2.5×10^5 viable cells/ml; 24 hr cultures. Labeling time, 60 min.

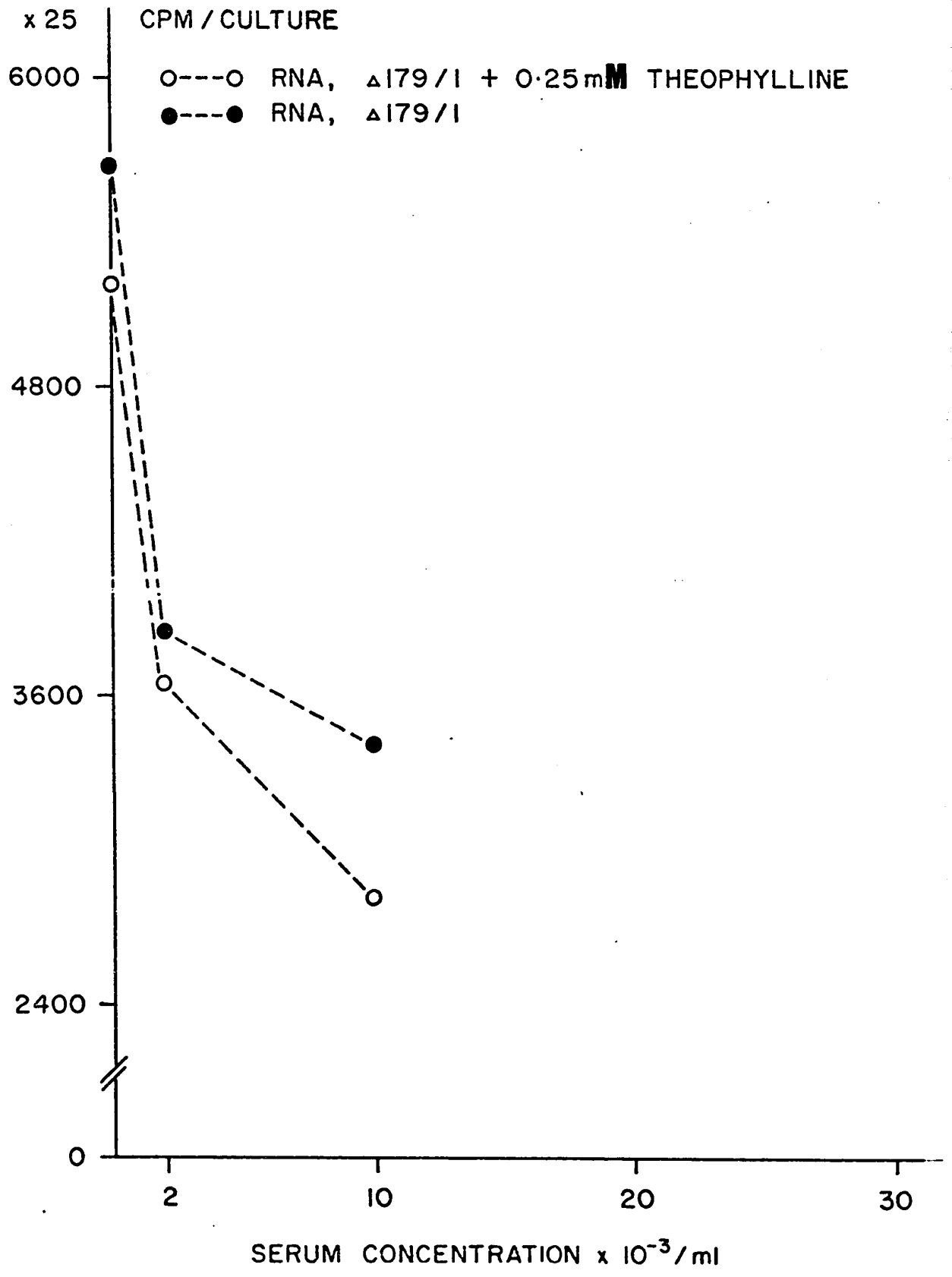
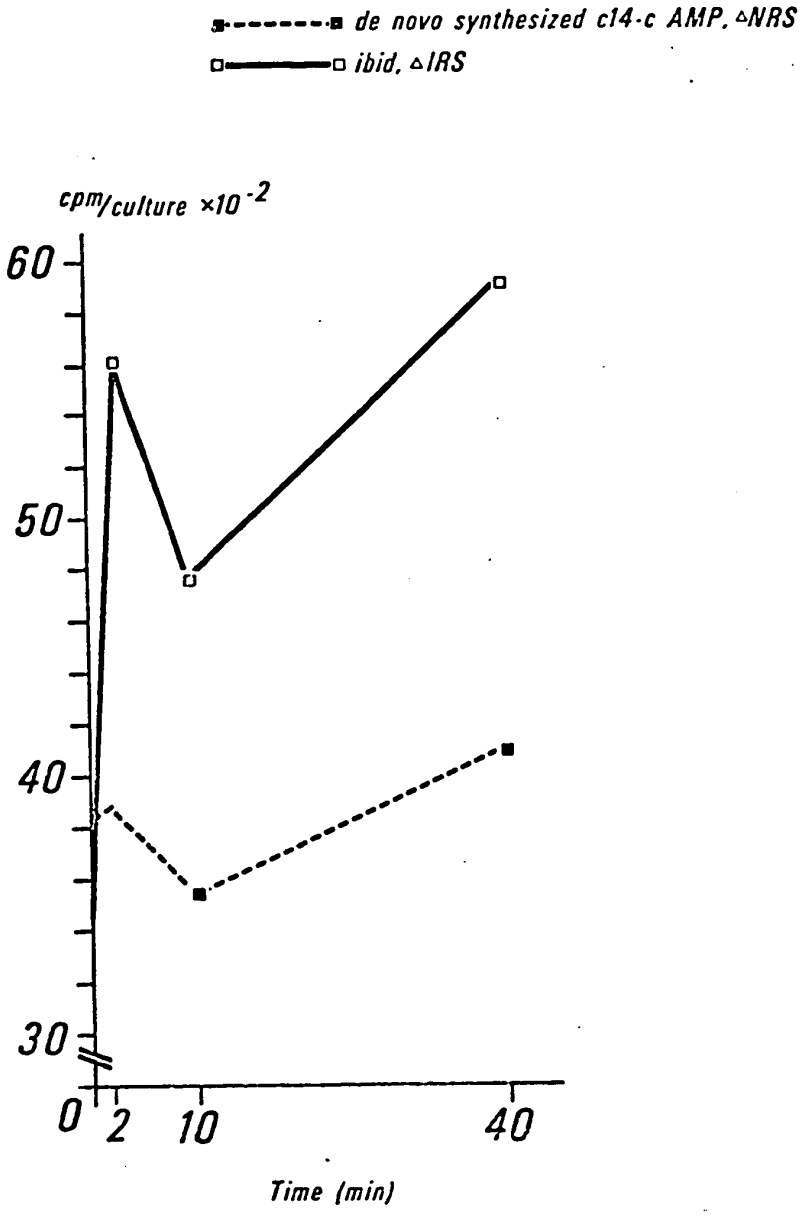

x 25
CPM / CULTURE
6000
4800
3600
2400
0
o---o RNA, Δ179/1 + 0·25mM THEOPHYLLINE
●---● RNA, Δ179/1
2
10
20
30
SERUM CONCENTRATION x 10^{-3}/ml

Figure 14 : Time effects of antiserum on cyclic AMP formation by L5178YR cells. 2.5×10^7 viable cells/culture(1 ml); separated by AG50W-X2(H^+) chromatography, barium sulfate adsorption and Whatman 3 MM chromatography.


■--------■ de novo synthesized c14-c AMP, △NRS
□——————□ ibid, △IRS
cpm/culture ×10⁻²
60
50
40
30
0
2
10
40
Time (min)

incubation.

The slow rate of the inhibitory processes and the previous finding that energy sources such as glucose and succinate, although not malate, were able to partially alleviate the harmful effect of antisera (Yang & Vas, 1970a) suggested that some indirect mechanisms, triggered by the primary antigen-antibody reactions at the cell surface, might be important. Dumonde et al. (1961) were able to observe histochemical changes such as a marked activation of lysosomal acid phosphatase and the dissociation of intracellular lipid-protein complexes even though the antibodies were shown by the fluorescent antibody technique to be located exclusively on the cell surface. Timbury (1963), when studying the mechanism of the inhibitory effects of anti-human amnion cell serum on the intracellular replication of Coxsackie A9 virus in amnion cells, found that the relationship between antiserum concentration and plaque inhibition is exponential except at very high doses of antiserum. He concluded that the inhibitory reaction between antiserum and the amnion cells is of first-order kinetics or a one-hit reaction. That is to say that one antibody molecule is apparently capable of inhibiting one cell or perhaps a part of a cell, such as a factory or entry site. He further speculated that antibody may combine with cell surface antigens disturbing some cellular process which is essential for the replication of the inhibited virus.

Metabolic derangement triggered as a result of the reaction between cell antigen and antibody seems to be one of the major factors

in both the present system and that of Timbury. Timbury had first speculated (1963) that antibody might stimulate the cells to produce interferon or some other inhibitory proteins but this has not been substantiated by his recent study (Timbury, 1969), in which he found that actinomycin D did not prevent the inhibitory action of antiserum on virus replication. This suggests that antiserum to host cells does not act by stimulating the cell to produce interferon or other inhibitory proteins.

The argument which might be made, in view of the recent finding of Grant et al. (1970) on the possible production and release of antibodies and complement-like substance by immune lymphocytes, is that the slow killing effect is due to the production of some heat labile components of complement by the cell. But this is most unlikely because the cell is of DBA/2 mouse origin which is known to be genetically deficient in C'5 (Rosenberg & Tachibana, 1962; Herzenberg et al., 1963; Cinader, Dubiski & Wardlaw, 1964; Nilsson & Müller-Eberhard, 1967). In a complement mediated system, Fell et al. (1968) have shown that complement components 1 to 6 at least were involved in producing the effect of complement and antibodies on embryonic cartilage and bone. It also has been well documented that all nine components of complement are needed for immune hemolysis (Müller-Eberhard, 1968) and for immune cytolysis of human amnion cells (Ross & Lepow, 1960).

As for the nature of antibody in the antisera, the injection procedure has produced substances in the serum which fulfill certain

basic criteria for antibodies. As a result of injection, reacting substances which are not present in pre-immune sera are produced. These substances are effective against the cells in fairly high dilutions, especially in cytotoxic activity in the presence of complement. The inhibitory activity of the antiserum could be absorbed by the cells and eluted from them. The ability of the antibody to absorb differentiates it from ablastin (Taliaferro, 1963); the two have many other common characteristics.

The activity of the antisera seems to reside mainly in the IgG fraction. In this research the immunization schedule of Levey and Medawar (1966) was adapted. These workers have reported high activity in IgG fractions (James and Medawar, 1967). In order to give some idea of the property of the antibodies, the heat resistance method was used (Locke & Segre, 1965; Pike, 1967). Even at much lower concentrations than the conventional 0.1 M, 2-mercaptoethanol was found to be too toxic for the cells and therefore, could not be used for differentiating IgG from IgM (Schur & Christian, 1964; Jakobsen, 1970). Furthermore, it has been reported that after 2-mercaptoethanol treatment, 7S rabbit antibodies to organ extracts suffered a complete loss of complement-fixing ability and a marked destruction of PCA activity (Brinckerhoff, Brown & Rose, 1970). Acid treatment could not be used because Anderson, James and Woodruff (1968) found that this treatment of anti-lymphocytic IgG results in a loss of its in vitro cytotoxic activity and its in vivo immunosuppressive activity.

The present experiment fulfills Sutherland's three postulates and thus substantiates the hypothesis that cyclic AMP is one of the major second messengers in the system. First of all, cyclic AMP is able to mimic the effect of the antiserum; secondly, theophylline, which is the specific inhibitor of the phosphodiesterase which degrades cyclic AMP, potentiates the action of the antiserum; and lastly, the antiserum stimulates an increase in the level of intracellular cyclic AMP. Cyclic AMP appears to be one of the major agents which initiate the sequence of events observed in this study. All the metabolic changes observed may be secondary effects which follow a primary injury by many hours.

Although a definite increase in cyclic AMP could be demonstrated after the cells were stimulated with the antiserum, the amount of cyclic AMP found is small. This low level may be of regulatory significance for HeLa cell adenyl cyclase activity has been reported to be low also (Makman, 1970) and HeLa cell growth has been shown to be inhibited by cyclic AMP (Ryan & Heidrick, 1968; Heidrick & Ryan, 1970). Of course, the low cyclic AMP level could also be due to the methods available for its measurement. Cyclic AMP is present in mammalian tissues in very low concentrations (0.2-1.5 mu moles per gm); the assay involves a variety of complex enzymatic reactions and initially requires the separation of cyclic AMP from other nucleotides by chromatographic techniques. Furthermore, the rather high serum concentration in the test system might degrade cyclic AMP since Steiner et al. (1969) have found that the rabbit

serum contains phosphodiesterase activity. The highly specific immunoassay method developed by Steiner et al. (1969) eliminates the need for prior chromatographic separation of cyclic AMP from other tissue nucleotides. However, the assay method involves the use of goat anti-rabbit immunoglobulin and this introduces another complicating factor in a system which contains rabbit serum. Further modification is needed for use in the present system.

During the preparation of this manuscript, articles by Colobert and Lagarde (1970) and Ishizuka, Gafni and Braun (1970) appeared further substantiating the hypothesis that cyclic AMP is one of the major mediators of the cell growth inhibition. Colobert and Lagarde demonstrated an increase in cyclic AMP levels in lymphocytes stimulated with anti-lymphocytic serum and Ishizuka et al. gave indirect evidence for the possible mediation of cyclic AMP in stimulating antibody formation by antigen. Mozsik (1969) offers a good explanation of how an increase in cyclic AMP levels and a decrease in energy production could coexist. He clearly demonstrated that the products of adenyl cyclase activity (cyclic AMP and adenosine 5'-monophosphate) directly inhibit a specific ATP-ase in a Na^+-K^+-dependent active transport system. In addition, he was able to show that drug stimulation of adenyl cyclase is associated with the blocking of the Na^+-K^+-dependent ATP-ase activity so the active transport ATP-ase system and the adenyl cyclase system must be separate.

The probability that antibody causes conformational changes

in the cell membrane or in its enzymes (Yang & Vas, 1970a), thereby activating membrane-bound adenyl cyclase has been also substianted by the dramatic results of Ellory and Tucker (1969). They used antibody against a specific antigen, m, which is found in LK cells (low potassium type sheep RBC) but absent in HK cells (high potassium type sheep RBC) to sensitize LK cells. The sensitization effectively conferred on the LK cells the properties of the HK cells. Because the antiserum does not remove the m antigen from the cell, Ellory and Tucker postulated that its position or conformation must be changed. It seems likely that the m antigen blocks the potassium pump either directly by allosteric inhibition of the potassium sites, or indirectly by conformational alteration of the enzyme-complex within the membrane.

Involvement of cyclic AMP in diversified systems such as hormone-target cells (Sutherland, 1970), sperm-egg membranes (Castanda & Tyler, 1968), and antigen-antibody interactions seems to reflect well the recent statement of Weiss (1968). " The

> immunological interactions (would) merely represent one special manifestation of a universal principle of steric matching or template-anti-template operating (or seem to operate) in diverse biological phenomena in areas seemingly as disparate as development, immunology, wound-healing, endocrine regulation, growth control, and the functioning of the nervous system."

Thus, the responses, stimulatory or inhibitory, of cells towards the same agent reflect their physiological states and their degree of differentiation or " determination ". This is exemplified by the responses of lymphocytes and lymphoblasts to anticellular antibodies (Gräsbeck et al., 1963; Yang & Vas, 1970a), phytohemagglutinin (Nowell, 1960; Yang et al., 1967) and cyclic AMP (MacManus & Whitfield, 1969; Yang & Vas, 1970b; Hirschhorn, Grossman & Weissmann, 1970). Recent papers by Manski and Ehrlich (1970) further substantiate this interpretation. They have demonstrated that the normally resistant rabbit corneal epithelium and endothelium become sensitive to cytotoxic antibodies after an " activated state " is induced in these tissues, that is, during cellular regeneration after injury.

5. Summary

The time-course study of the inhibitory effects of antiserum on the metabolic events in mouse leukemia L5178YR cells provided evidence that the rate of RNA synthesis decreases significantly at 4 hr, while the rates of protein and DNA syntheses showed demonstrable changes only after 6 to 8 hr, and 8 hr, respectively.

Cyclic AMP mimics the action of the antiserum and theophylline, which is a specific inhibitor of the phosphodiesterase, potentiates it. Stimulation of de novo synthesis of cyclic AMP by antiserum further substantiates the hypothesis that antibodies, in the absence of complement, inhibit the growth of cells indirectly

through second messengers such as cyclic AMP.

CHAPTER III. GENERAL CONCLUSIONS

An in vitro system consisting of rabbit serum-containing medium, a medium-adapted subline of L5178Y cells and rabbit antiserum was used to study the effects of antibodies, in the absence of complement, on cells in culture.

Heat-inactivated antiserum can inhibit cell growth and colony formation in soft agar, and kill the cells. These actions occur slowly and in a certain sequence of metabolic events. Studies using radioactive precursors showed that RNA synthesis is inhibited after 4 hr, but the effects on the rates of protein and DNA syntheses become demonstrable only after 6 to 8, and 8 hr, respectively. Maximal inhibition is obtained after 24 to 48 hr.

Two hr after the addition of certain antisera, there is a transient increase both in viable cell counts and in uptake and incorporation of ^{14}C-2-glycine. This is probably due to direct " stimulation " of noncycling G2 cells into mitosis by the antisera.

During the entire experimental period, i.e., up to 4 days, a small portion of the cell population remained viable. Many of these cells looked much larger and their rate of incorporation, but not of uptake, of ^{14}C-2-glycine per 10^6 viable cells was much higher than that of the control. Cultures treated with antiserum recovered if fresh medium without antiserum was added at 24 to 48 hr.

The soft agar technique was then used to clone antibody-resistant cells and studies made on their susceptibity to antiserum. Unexpectedly, the cells derived from resistant clones showed a degree

of sensitivity to antiserum similar to that of the parent population in both the ability to form colonies in soft agar and to grow and incorporate radioactive precursors into RNA and protein.

All the antisera showed agglutination. However, agglutination was shown not to be the essential cause of cell growth inhibition. Antiserum inhibited colony formation in soft agar even when it was placed over the cell-containing agar layer to prevent agglutination.

Energy sources such as glucose and succinate were all shown to protect the cells, at least partially, against the harmful effects of antiserum, suggesting that the substrate uptake and/or the energy generating mechanism of the cell became inefficient after its contact with the antiserum. Thus, in the absence of complement, there is no preferential inhibition of the glycolytic pathway, as Flax has proposed for his complement-mediated system. Moreover, malate was found to be without effect. These observations together with the evidence for the slow nature of the inhibitory process of the antiserum on the cells suggests that some indirect mechanism, triggered as a result of the primary antigen-antibody reactions on the cell surface, is responsible.

Reports by others on the relationship of glucose, malic enzyme and nicotinic acid to cyclic AMP, on the growth inhibitory effects of various tissue culture cell lines, and on the activation of adenyl cyclase at fertilization suggest that cyclic AMP is one of the major mediators of the system.

Cyclic AMP was found to mimic the inhibitory effects of antiserum on the metabolic events in the cells. Theophylline, a specific inhibitor of the phosphodiesterase, potentiated the action of the antiserum. Stimulation of de novo synthesis of cyclic AMP by antiserum further substantiates the indirect mode of action of antibodies through second messengers such as cyclic AMP.

CLAIMS TO ORIGINALITY

This investigation has contributed the following original knowledge to the action of anticellular antibodies, in the absence of complement, on mammalian cells in culture.

1. It has been established that heat-inactivated antiserum can both inhibit cell growth and colony formation in soft agar, and kill the cells. These actions occur slowly and the accompanying metabolic events, in sequence; that is, RNA synthesis is inhibited first and protein and DNA syntheses only 2 hr later.
2. Energy sources such as succinate and glucose were found to protect the cells, at least partially, against the inhibitory effects of antiserum.
3. Cyclic AMP was shown to affect the growth of the cells and the metabolic events in sequence. A mechanism of cell growth inhibition by cyclic AMP through its preferential action on initiation of RNA transcription and DNA replication is proposed. Indirect mode of action of antibodies mediated by second messengers, such as cyclic AMP is proposed.

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