

# HUMORAL IMMUNOSTIMULATION

## IV. Role of Complement\*

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Previous studies in our laboratory have demonstrated that, under appropriate conditions, in vitro tumor cell growth and metabolism are stimulated by antitumor antibodies as evidenced by increased cellular incorporation of radioactive nucleosides (references 1-3, and footnote 1 and 2), DNA synthesis,<sup>1, 2</sup> and cell growth (reference 4 and footnote 1). Stimulation was obtained following exposure of 2,4, 6-trinitrophenyl (TNP)-substituted HeLa, HEp-2, and L cells to highly purified anti-TNP rabbit antibody or HeLa, HEp-2, L, and MOPC-315 cell lines to their respective whole-cell rabbit antibodies or isolated IgG fractions (references 4 and 5, and footnotes 1 and 2). The effect of antibody was dose dependent with high concentrations inhibiting cell growth and lower concentrations stimulating cell growth (reference 4 and footnote 1). On the basis of these observations we suggested that part of the enhancing effect of antibodies on tumor cell growth in vivo might involve a direct stimulatory effect of the antibody on tumor cell metabolism. Since complement (C) is present in vivo and our earlier in vitro studies had been performed with heat-inactivated serum, it became important to investigate the influence of C on the phenomenon. In the present study we will present evidence that C greatly augments immunostimulation in vitro with utilization of both classical and alternate pathways through C3. A preliminary report of this work has appeared (6).

### Materials and Methods

*Cell Line.* Mouse L cells (7), obtained from Grand Island Biological Corp., Grand Island, N. Y., were used throughout. The cells were maintained in plastic T flasks (Falcon Plastics, Div. of

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<sup>1</sup> Shearer, W. T., G. W. Philpott, and C. W. Parker 1975. Humoral immunostimulation. II. Increased nucleoside incorporation, DNA synthesis, and cell growth in L cells treated with anti-L-cell antibody. *Cell. Immunol.* In press.

<sup>2</sup> Shearer, W. T., H. J. Wedner, G. W. Philpott, and C. W. Parker. 1975. Humoral immunostimulation. III. Cross-reactivity and specificity with cell lines treated with anti-L-cell antibodies. Manuscript submitted for publication.

BioQuest, Oxnard, Calif.) in a nutrient medium (NM)<sup>3</sup> containing Eagle's minimum essential medium with glutamine plus 10% fetal calf serum (FCS). NM prepared with heat-inactivated (56°C for 30 min) FCS (hNM) was used in some experiments. A 5% CO<sub>2</sub>-humidified air atmosphere at 37°C was used for cell incubation. Difco trypsin (1:250; Difco Laboratories, Detroit, Mich.) which has been shown to possess 740 benzoyl-L-arginine ethyl ester U/mg of enzyme<sup>1</sup> was used to harvest the cells (see below).

*Antiserum.* Antiserum was raised in rabbits by immunization with an L cell-complete Freund's adjuvant emulsion as previously described (reference 4 and footnote 1). The animals received  $2 \times 10^6$  cells on three occasions each 2 wk apart. The first two injections of cells were in complete Freund's adjuvant; the last injection of cells was in 0.01 M phosphate, pH 7.4, 0.15 M NaCl (PBS). Control rabbit serum (CR) was obtained by injection of complete Freund's adjuvant alone (reference 4 and footnote 1). Naive rabbit serum (animals unimmunized) was used for some experiments. Sera were obtained at various times after initial immunization. As a rule, they were heat inactivated at 56°C for 30 min. All sera were sterile filtered.

*Reaction of Cells with Antibody.* Logarithmically growing L-cell monolayers were washed with Ca<sup>++</sup>, Mg<sup>++</sup>-free Hanks' balanced salt solution, exposed to 0.05% trypsin for 3-5 min, scraped off the T flask, washed with either NM or hNM, pipetted into single cell suspensions, and plated in 1-ml vol in 12 x 75 mm plastic tissue culture tubes (Falcon Plastics, Div. of BioQuest) at a cell density of  $5 \times 10^4$ /ml. Small volumes of PBS alone or PBS containing varying amounts of serums were added to the cells.

*Sources of C.* Purified individual C components were obtained from Cordis Laboratories, Miami, Fla. Normal sera containing C were obtained from fetal calves, noninbred, National Institutes of Health multipurpose guinea pigs (NGPS), humans (NHS), BALB/C mice (NMS), and New Zealand white rabbits (NRS). C-deficient serums were obtained from guinea pigs congenitally deficient in the fourth component (C4D) (8, 9), guinea pigs depleted of the third through ninth C components [(C3-C9)D] (10), human serum congenitally deficient in C1r (C1RD) (11), a human congenitally deficient in C2 (C2D) (12), a human with hereditary angioedema which had no detectable C2 and less than 0.5% normal level of C4 (C2,4D) (13), a human congenitally deficient in C5 (C5DHS) (14), mice congenitally deficient in C5 (C5DMS) (15), and rabbits congenitally deficient in C6 (C6DRS) (16).

*Assay of C Activity and C Components.* The methods for the preparation of C reagents and methods for the hemolytic titration of CH<sub>50</sub>, C1, C2, C5, and C3-C9 complex have been previously described (9, 17-20). Partially purified C proteins were either prepared in our laboratory by standard techniques (8, 9) or obtained from Cordis Laboratories.

C4 hemolytic titrations were performed utilizing C4D (8, 9). This serum contains an excess of all C components except C4. A detailed investigation of this new assay system has been published (21). Hemolytic activity of C6 was determined by a similar method in which the ability of the test serum to reconstitute C activity to C6DRS was employed (22, 23).

The preparation of guinea pigs depleted of the late-acting C components C3-C9 by cobra venom factor (Cordis Laboratories) has been previously published (10). 20 h after intravenous injection of 20 U of cobra venom factor/100 g the animals were sacrificed by exsanguination. The C3-C9 hemolytic titer was determined on this serum, and, if the titer was less than 0.5% of normal, the serum was subsequently employed in *in vitro* studies.

*Immunostimulation Assay.* Previous studies showed that the stimulation of cellular incorporation of radioactive nucleosides was maximal (up to 200-fold) 24-72 h after exposure to antibody (reference 4 and footnote 1). Virtually all of the radioactivity was in DNA.<sup>1</sup> This was true for [*methyl*-<sup>3</sup>H]thymidine and for [<sup>125</sup>I]-5-iodo-2'-deoxyuridine ([<sup>125</sup>I]UdR). [<sup>125</sup>I]UdR (Amersham/Searle Corp., Arlington Heights, Ill., original sp act 200-2,000 mCi/mmol) was added to the cells at 48 or 72 h

<sup>3</sup> *Abbreviations used in this paper:* AL, anti-L-cell antiserum; C1RD, human serum lacking C1r; C2D, human serum lacking C2; C2,4D, human serum lacking C2 and C4; (C3-C9)D, guinea pig serum lacking C3-C9; C4D, guinea pig serum lacking C4; C5DHS, human serum lacking C5; C5DMS, mouse serum lacking C5; C6DRS, rabbit serum lacking C6; CR, control rabbit serum; FCS, fetal calf serum; hNM, NM containing heat-inactivated FCS; [<sup>125</sup>I]UdR, [<sup>125</sup>I]-labeled 5-iodo-2'-deoxyuridine; NHS, normal human serum; NM, nutrient medium; NMS, normal mouse serum; NGPS, normal guinea pig serum; NRS, normal rabbit serum; PBS, 0.01 M phosphate, pH 7.4, 0.15 M NaCl; PHA, phytohemagglutinin; RS, control rabbit serum and anti-L-cell antiserum; SR, stimulation ratio.

of incubation. 24 h later the cells were harvested and the residual cellular radioactivity was determined (reference 4 and footnote 1). The stimulation ratio (SR) was calculated by the mean counts per minute of experimental cells divided by that of PBS control cells.

*Consumption of Guinea Pig C4.* Cells were trypsinized for 3–5 min as described above and plated into 12 × 75-mm tissue culture tubes in 1 ml of hNM at a cell density of  $8 \times 10^6$ /ml. PBS, CR, or rabbit anti-L-cell antiserum (AL) was added in various final dilutions before the addition of NGPS (0.25% vol/vol). After incubation at 37°C for various time periods, the supernates were aspirated from replicate centrifuged samples and stored at –80°C until assayed for guinea pig C4 levels.

*Human C3 Fluorescence Microscopy.* Cells were plated in hNM at a  $5 \times 10^6$  cell density/ml and incubated with PBS, CR (1:2,000 final dilution), or rabbit anti-L-cell antiserum (AL) (1:2,000 final dilution). 10  $\mu$ l of fresh human serum were added to all conditions (in 1 ml) and the cells were incubated for 30 min at 37°C, washed three times, and cooled to 4°C. The cells were incubated with a 1:60 dilution of monospecific goat antihuman C3 (Hyland Div., Travenol Laboratories Inc., Costa Mesa, Calif.) for 30 min at 4°C, washed, incubated with a 1:60 dilution of rhodamine-conjugated rabbit antigoat IgG antiserum (Cappel Laboratories, Inc., Downingtown, Pa.) for 30 min at 4°C, washed, and examined with a Leitz Ortholux fluorescence microscope with an episcopic light attachment (E. Leitz, Inc., Rockleigh, N. J.). All sera except the NHS were heat inactivated.

*C-Mediated Cytotoxicity.* Cells were prepared in single cell suspension and plated in 1 ml of hNM at  $2 \times 10^6$ /ml. Small volumes of PBS, CR (1:10 final dilution), AL (1:10 final dilution), guinea pig sera (10% vol/vol), and guinea pig C component C3 (100 U) were added to the appropriate tubes. The cells were incubated at 37°C for 60 min, washed, plated in 1 ml of hNM containing [ $^{125}$ I]UdR (0.05  $\mu$ Ci/ml), incubated for 24 h, washed, and counted for residual gamma radioactivity.

## Results

*Studies in Heated and Unheated Sera.* In the presence of unheated FCS and high dilutions of antibody there was an increase in both the maximum stimulation ratio and in the dilution of antiserum that produced a detectable increase in uptake of [ $^{125}$ I]UdR. Fig. 1 demonstrates this augmentation of nucleoside incorporation into antibody-treated L cells when the FCS in the medium was unheated (column B as compared with column A). In more concentrated antibody solutions (1:20) inclusion of unheated FCS in the medium lowered the stimulation ratio to below 1.0, indicating cell killing. The augmentation of both cytotoxicity and stimulation was especially dramatic in antisera obtained late in the course of immunization (from unboosted animals). Similar C-dependent effects were seen with the Ig and IgG fractions isolated from the antisera (data not shown).

It seems likely that the cytotoxic effect obtained at high concentrations of antibody in the presence of unheated FCS involved classical antibody C-dependent cell lysis. The cytotoxic effect with antiserum was in marked contrast to what was observed with an equivalent amount (1:20 dilution) of naive or CR where stimulation of nucleoside incorporation was obtained. This may be a nutritional effect since the medium contains only 10% FCS and the addition of 5% rabbit serum raises the protein content of the medium considerably. Alternately the stimulatory properties of the 1:20 dilution of naive or CR might involve the nonspecific adherence of aggregated rabbit Ig on the cell surface with activation of nucleoside incorporation. Whatever the explanation, nonspecific nutritional effects can not explain the stimulation with anti-L-cell antibody since stimulation of nucleoside incorporation occurred at antiserum dilutions up to 1:200,000 (Fig. 1 B, 2). The only dilution of CR that produced a stimulatory effect at a greater than 1:20 dilution was found in B,3 (Fig. 1). This may represent a

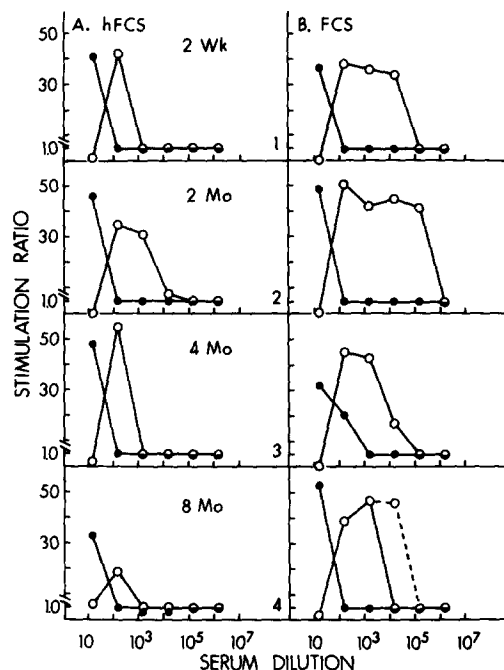


FIG. 1. Stimulation of nucleoside uptake in L cells treated with RS: Effects of fresh serum and time after immunization. A column (vertical): experiments with heat-inactivated FCS(hFCS) in the medium. B column: experiments with FCS in the medium. Rows (horizontal) 1, 2, 3, and 4 indicate sera harvested 2 wk, and 2, 4, and 8 mo after immunization, respectively. The dashed line indicates the RS obtained using antiserum 1 wk after boosting the animals (8 mo after initial immunization) with L cells without adjuvant. Cells were cultured in either hNM or NM in the presence of either PBS, CR (closed circles), or AL (open circles) at the final dilutions indicated. All determinations were performed in triplicate; PBS controls had 300–600 cpm. Naive rabbit serum produced a stimulative effect at 1:20 dilution identical to CR.

natural antibody or a fortuitous, immunologic cross-reaction between cells and *Mycobacterium tuberculosis* in the adjuvant.

If the factor in unheated FCS which enhances cytotoxicity is C then it should be possible to show that unheated sera from other species can also promote the response. In accord with this possibility when both the RS and the FCS in the medium were heated the stimulatory effect of antibody was lost almost entirely (even though some specific cytotoxicity remained as compared to CR) (Fig. 2 D). When the rabbit antibody was unheated and the FCS was heated the response was partially restored (Fig. 2 B) indicating that the stimulating factor is present in the rabbit serum as well as in the FCS. Moreover, as will be shown below, NGPS, NMS, and NHS also possess this property.

*Studies with Sera Deficient in C1, C4, C2, and C3-C9 C Components.* Guinea pig C component C4 as well as the later components C3-C9 were found to be quite important for augmentation of immunostimulation. In antiserum obtained late after immunization almost no augmentation of immunostimulation was seen with either the C4D or the (C3-C9)D (maximal stimulation ratios 4.96 and 2.37,

respectively, as compared to 152 for NGPS at an antiserum dilution of 1:200 (Table I). With earlier antisera the response to (C3-C9)D was again essentially absent but the C4D was partially active provided high concentrations of antibody were used (Table II). Thus, it appeared that not only was C important in the stimulation by antibody but that both the alternate and classical pathways could be utilized depending on the antiserum concentration and the experimental conditions. Whether this is an effect of antibody concentration per se or the class of antibody participating in the response remains to be elucidated.

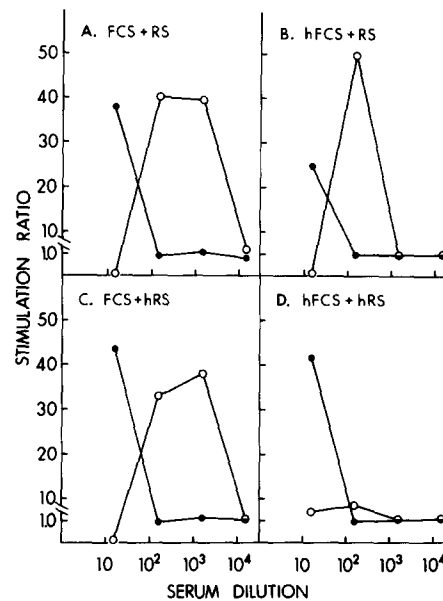


FIG. 2. Stimulation of nucleoside uptake in L cells treated with RS harvested 8 mo after immunization: Effects of fetal calf C and rabbit C. A, unheated FCS in the medium and unheated rabbit sera (RS); B, heat-inactivated FCS and unheated rabbit sera; C, unheated FCS and heat-inactivated rabbit sera; and D, heat-inactivated FCS and rabbit sera.

TABLE I  
Effect of Guinea Pig C4 and C3-C9 Upon Immunostimulation: Results with a Late Antiserum of Low Titer

Sera added to hNM . . . . .	None		NGPS		C4D		(C3-C9)D	
	cpm $\pm$ SE	SR	cpm $\pm$ SE	SR	cpm $\pm$ SE	SR	cpm $\pm$ SE	SR
PBS	295 $\pm$ 93	1.00	263 $\pm$ 38	1.00	275 $\pm$ 38	1.00	295 $\pm$ 65	1.00
CR 1:200	258 $\pm$ 42	0.87	588 $\pm$ 263	2.24	228 $\pm$ 38	0.83	251 $\pm$ 40	0.85
CR 1:2,000	162 $\pm$ 24	0.55	168 $\pm$ 46	0.64	327 $\pm$ 76	1.19	248 $\pm$ 41	0.84
CR 1:20,000	192 $\pm$ 38	0.65	151 $\pm$ 19	0.57	271 $\pm$ 28	0.99	175 $\pm$ 48	0.59
AL 1:200	541 $\pm$ 113	1.83	39,950 $\pm$ 685	152	652 $\pm$ 22	2.37	1,464 $\pm$ 41	4.96
AL 1:2,000	384 $\pm$ 103	0.78	37,567 $\pm$ 177	143	570 $\pm$ 4	2.07	478 $\pm$ 58	1.62
AL 1:20,000	557 $\pm$ 94	1.89	1,678 $\pm$ 823	6.38	342 $\pm$ 16	1.24	329 $\pm$ 126	1.12

Cells were incubated in the presence of various final dilutions of CR or AL harvested 8 mo after immunization. Guinea pig sera from animals described in the Materials and Methods were added, i.e., 5  $\mu$ l to 1 ml: 0.5%. The results are expressed as the mean of triplicate determinations (cPM)  $\pm$  standard error of the mean (SE).

Several human sera also were tested for their ability to amplify stimulation of nucleoside incorporation by antibody (Table III). The serum from a patient with hereditary angioedema which was almost completely deficient in C2 plus C4 produced much less augmentation than NHS (maximum stimulation ratios of 8.2 and 139, respectively). The serum from a patient with a total C1RD produced stimulation of nucleoside uptake although it was less potent in this regard than NHS (Table III). This patient has C1<sub>q</sub> and C1<sub>s</sub> present in his serum and thus has 1-2% of the normal level of functional C1 (about 2,000 u/ml). At 0.2% of C1RD there was very little stimulation but at 0.5% there was a considerable stimulation effect. This implies that free C1<sub>s</sub>, although highly inefficient, can stimulate cellular incorporation of nucleoside in this system. Similarly, this concentrated serum has some lytic activity in other systems.

*Studies with Sera Deficient in C5 and C6 C Components.* The ability of C5- and C6-deficient sera to promote immunostimulation was examined in C5DHS, C5DMS, and C6DRS (Fig. 3). C5DHS promoted the increased uptake of radioactive nucleoside as well as the NHS. The responses to C5DMS and NMS were very similar. Judging from the results with C6DRS C6 also was nonessential for C enhancement of immunostimulation.

The possibility that augmentation of nucleoside incorporation in the presence of C5DHS, C5DMS, or C6DRS was due to C5 or C6 in the hNM was considered, and we therefore determined the level of C5 and C6 in the hNM by functional assays (Table IV). The values for C5 and C6 in both hNm and NM were very low. Thus it was unlikely that either C5 or C6 was being activated to a major extent when cells interacted with antibody, even when unheated FCS was present. As further evidence against the presence of C5 in hNM the addition of heat-inactivated FCS (1:1 dilution) did not restore any functional hemolytic units to the C5DHS. Table IV also shows that heat inactivation of the FCS reduced the C4 level in hNM to an almost undetectable level. The C3 and C4 levels of the C5DHS were normal, but the total hemolytic C level was zero.

*Reconstitution Experiments with Normal Serum or Purified C Components.* Restoration of augmentation of immunostimulation was performed by the addition of a small amount of normal serum to a C component-deficient serum in the presence of antibody and by the addition of the purified C component itself. For purposes of clarity, the restoration effects are presented for only the 1:2,000 dilution of antiserum, although restoration was observed at higher and lower dilutions of antiserum. Thus augmentation of immunostimulation with C4D could be restored with a small amount of NGPS in a dose-related fashion (Fig. 4). These amounts of NGPS did not similarly stimulate nucleoside incorporation of identical conditions in hNM alone.

Purified guinea pig C3 restored the ability of (C3-C9)D to enhance immunostimulation. Fig. 5 shows the specificity of the restoration effect. C3 restored immunostimulation to (C3-C9)D and not to C4D serum. The purified component had little effect upon the immunostimulation properties of hNM or NGPS. In experiments not shown we demonstrated a dose-response effect of C3 added to (C3-C9)D. To insure that the restoration of (C3-C9)D was not a nonspecific effect of purified C component we attempted to restore the stimulation with C2 (human and guinea pig) but failed (Fig. 5).

TABLE II  
Effect of Guinea Pig C4 and C3-C9 Upon Immunostimulation: Results with IgG Isolated from An Early Antiserum of High Titer

Antibody added to hNM . . . . . None		NGPS		C4D		(C3-C9) D		
Conditions	cpm ± SE	SR	cpm ± SE	SR	cpm ± SE	SR	cpm ± SE	SR
<i>µg/ml</i>								
PBS	808 ± 113	1.00	643 ± 135	1.00	586 ± 110	1.00	579 ± 132	1.00
AL 33	792 ± 245	0.98	23,451 ± 1,575	36.5	25,363 ± 564	42.8	1,119 ± 119	1.91
AL 20	520 ± 26	0.65	22,943 ± 409	35.7	23,388 ± 76	40.0	1,143 ± 210	1.93
AL 13	881 ± 127	1.09	23,614 ± 544	36.7	25,430 ± 888	42.9	919 ± 114	1.59
AL 7	604 ± 120	0.75	19,448 ± 2,400	31.0	619 ± 200	1.03	580 ± 333	1.00

See legend to Table I. The final concentration of guinea pig serum was 0.5%. AL was harvested 2 mo after immunization.

TABLE III  
Effect of Human C1 and C2 Plus C4 Upon Immunostimulation

Sera added to hNM . . . . . None		NHS		C1RD		C2,4D		
Conditions	cpm ± SE	SR	cpm ± SE	SR	cpm ± SE	SR	cpm ± SE	SR
PBS	276 ± 36	1.00	181 ± 21	1.00	252 ± 34	1.00	247 ± 20	1.00
CR 1:200	154 ± 18	0.56	315 ± 91	1.74	209 ± 23	0.83	264 ± 33	1.07
CR 1:2,000	239 ± 54	0.87	197 ± 55	1.09	210 ± 23	0.83	256 ± 79	1.04
AL 1:200	1,184 ± 213	4.28	25,197 ± 711	139 (95.8)	643 ± 61	2.55 (82.8)	2,031 ± 102	8.20 (5.44)
AL 1:2,000	215 ± 25	0.78	27,445 ± 503	152 (143.8)	296 ± 11	1.17 (86.4)	282 ± 27	1.14 (1.57)

See legend to Table I. The antiserum was harvested 8 mo after immunization. The final concentration of human sera was 0.2%. The numbers in parentheses represent the antibody stimulation ratios obtained with 0.5% sera.

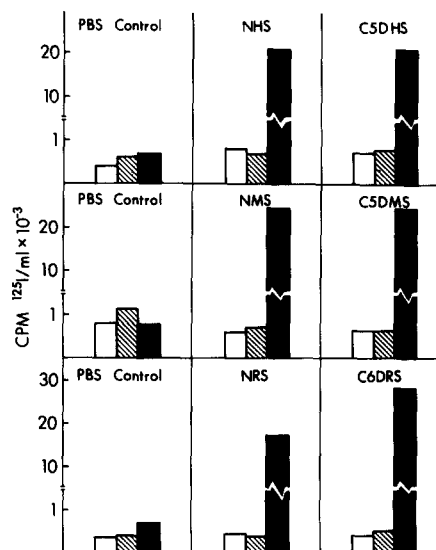


FIG. 3. Effects of C5 and C6 upon immunostimulation. Cells were cultured in hNM in the presence of small volumes of PBS (clear columns), a 1:2,000 dilution of CR (hatched columns), or a 1:2,000 dilution of AL (solid columns). Small volumes of either PBS (PBS control column) or other serums (0.2% final concentration vol/vol) were added to replicate sets of tubes. C5DMS was from SWR/J mice obtained from Dr. Philip D. Hoffsten (Department of Medicine, Washington University School of Medicine, St. Louis, Mo.) who has demonstrated (unpublished observations) that SWR/J mice do not have C5 in their blood. The method of analysis was lack of precipitation in double diffusion in agar gels; the antiserum was obtained by repeated intraperitoneal injections of serum from a B10 D2 New/J mouse (has C5) into a B10 D2 Old/J mouse (lacks C5) (15).

TABLE IV  
*C Activity and C Component Levels in Mediums and Sera*

	NM	hNM	NHS	C5DHS	C5DHS + hFCS (1:1)
C3 (mg/100 ml)*			101-188	162	
C4 (mg/100 ml)*			20-50	42	
C4 (effective hemolytic U/ml)	2.9	0.7			
C3 (effective hemolytic U/ml)	1.6	0.1	62,750	0	
C6 (effective hemolytic U/ml)	1.4	0.9			
Total hemolytic C (CH50 U)			175-250	0	0

\* Performed using Hyland Laboratories, Costa Mesa, Calif., radial immunodiffusion plates.

Since the addition of purified C3 to (C3-C9)D restored its immunostimulatory activity, we attempted to look at the ability of purified guinea pig C3 to restore cytotoxic activity (Fig. 6). Here using a high concentration of the late (8 mo) antiserum we were able to observe C-mediated cytotoxicity with NGPS (75% reduction in counts per minute under the conditions used), no cytotoxicity with (C3-C9)D by the addition of purified guinea pig C3. It was obvious, then, that C activation through C3 only was necessary for cyto stimulation but cytotoxicity required components acting later than C3.



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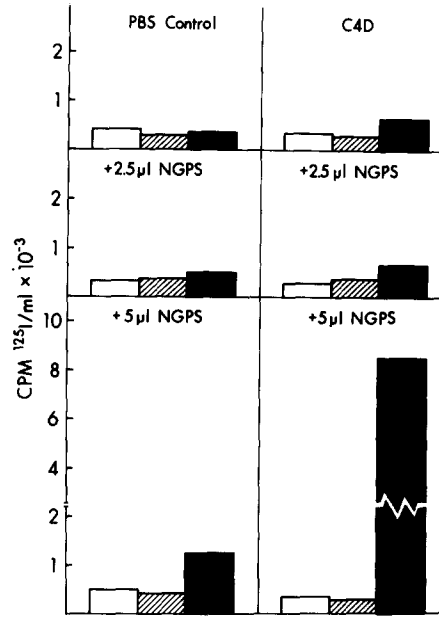


FIG. 4. Restoration of immunostimulation with C4D by addition of NGPS: dose-response effect. See legend to Fig. 3. Small volumes of either PBS (PBS control column) or C4D (C4D column) (0.1% final concentration vol/vol) were added to replicate sets of tubes. Finally, small volumes of NGPS were added to some of the replicate sets of tubes.

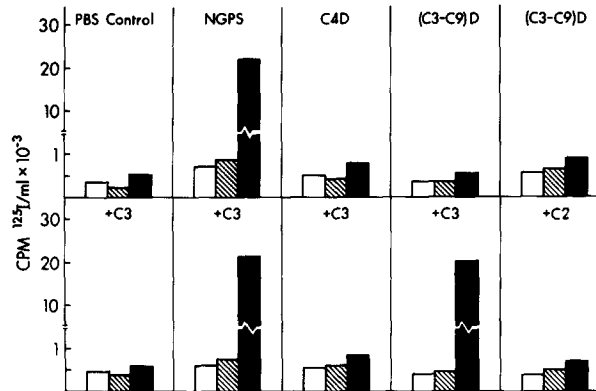


FIG. 5. Restoration of immunostimulation with (C3-C9)D by addition of guinea pig C3. See the legend to Fig. 4 for the general description of conditions and symbols. The final concentration of the guinea pig sera was 0.2%. Small volumes of highly purified guinea pig C components, containing 10 functional units, were added to some replicate sets of tubes. Human C2 also did not restore (C3-C9)D (data not shown).

Restoration was also studied with C2D. Purified human C2 restored augmentation of nucleoside incorporation into antibody-treated cells in the presence of C2D (Fig. 7). No augmentation was seen when C2 was added to antibody-treated cells in the absence of human sera or with NHS, C1RD, or C2,4D. Human or guinea pig C3 did not restore C2D.

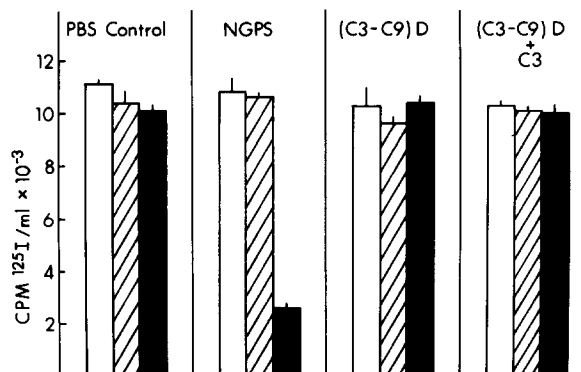


FIG. 6. C-mediated cytotoxicity: Attempted restoration of (C3-C9)D guinea pig serum with purified guinea pig C3. See Materials and Methods for description of procedure. Symbols are the same as those for Fig. 4 except the CR and rabbit antiserum concentrations were higher (1:10 final dilution). Attempted restoration of (C3-C9)D was made with 100 U of highly purified guinea pig C3. Results are expressed as the mean of triplicate determinations; the lines represent the standard error. Incubation with [ $^{125}$ I]UdR was from 0-24 h before harvesting and counting.

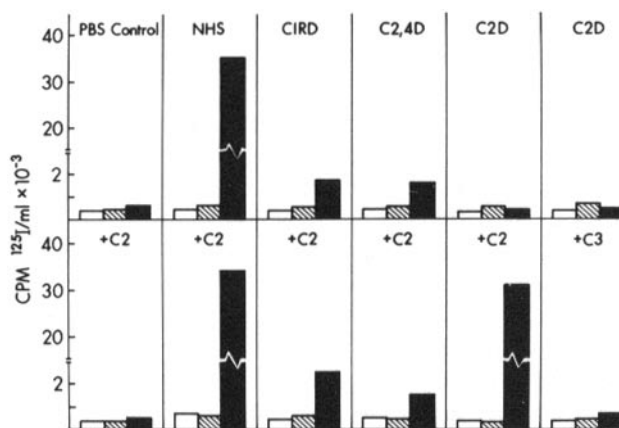


FIG. 7. Restoration of immunostimulation with C2D by addition of human C2. See the legend to Fig. 4 for the general description of conditions and symbols. The final concentration of guinea pig sera was 0.2%. Small volumes of highly purified human C components, containing 10 functional units, were added to some replicate sets of tubes. Guinea pig C3 also did not restore C2D (data not shown).

As additional evidence for the activation of C in the immunostimulation phenomenon we determined that there was a consumption of C4 during the early states (Table V). There was both a time-dependent and antibody dose-dependent consumption of guinea pig C4 during the first 12 h after exposure of cells to antibody.

*Fluorescence Microscopy Studies.* Fig. 8 shows a specific deposition of human C3 upon the membranes of cells treated with antiserum. In addition there was redistribution and cap formation of the adherent C3 indicating an active

TABLE V  
C4 Consumption During Immunostimulation

	0.5h	4h	12h
PBS	100 ± 1	104 ± 7	98 ± 3
CR 1:200	85 ± 8*	81 ± 12	69 ± 3‡
CR 1:2,000	105 ± 5	98 ± 9	89 ± 2
AL 1:200	68 ± 14‡	66 ± 0§	48 ± 2§,
AL 1:2,000	100 ± 7	80 ± 6*	65 ± 7‡,

The results are the means of quadruplicate (PBS) and duplicate (CR and AL) determinations of the functional C4 U/ml. Results are expressed as the mean ± standard error. There was some drop in C4 level with CR at the 1:200 dilution which may be related to nonspecific activation by aggregated Ig. For differences between experimental values and those of PBS controls at the same hour: \* $P < 0.05$ , † $P < 0.01$ , § $P < 0.001$ , || $P < 0.05$  from CR at the same dilution and hour.

cellular response to membrane perturbation. PBS control cells and cells treated with control serum did not show these effects.

### Discussion

At one time C was thought of as an agent which primarily mediated cell membrane lysis leading to the destruction of cells. More recently it has been recognized that C has other potent biological activities including effects on phagocytosis (24), lysosomal enzyme and histamine release (25, 26), vascular tone and permeability (27), chemotaxis (28), clot formation in some species (29), and bone resorption (30). The data presented in this report and elsewhere<sup>1</sup> suggest that C may have yet another biological function, namely, that of stimulation of cell growth and metabolism. It is not known whether the stimulatory effect of C is due to sublethal damage and attempted cellular repair or whether there is a more specific cell activation by C. Guttler demonstrated a C-dependent twofold increase in phospholipid biosynthesis in HeLa cells incubated with rabbit anti-HeLa IgG and guinea pig C (31). He assumed that a repair process was involved but there was no attempt to determine whether or not late C components were needed for the response. While our studies do not clarify the basis for the stimulation of phospholipid synthesis they do indicate that in the stimulation of cell growth late C components (C5-C9) are not required, suggesting that C effects on cell metabolism other than membrane lysis per se need to be considered.

Although the basis for the augmentation of immunostimulation in association with activation of the early C sequence is not presently clear, several explanations are possible. Since a number of activated C components have proteolytic activity, there may be a direct catalytic action of C on the cells. Proteolytic enzymes have been shown to stimulate mitogenesis in cultured peripheral lymphocytes and to enhance the response of these cells to phytohemagglutinin (PHA) (32). It has been suggested that proteolytic enzymes are normally activated or released into the medium during stimulation by PHA (33-35). A more general role for fibrinolytic enzymes in oncogenic transformation is

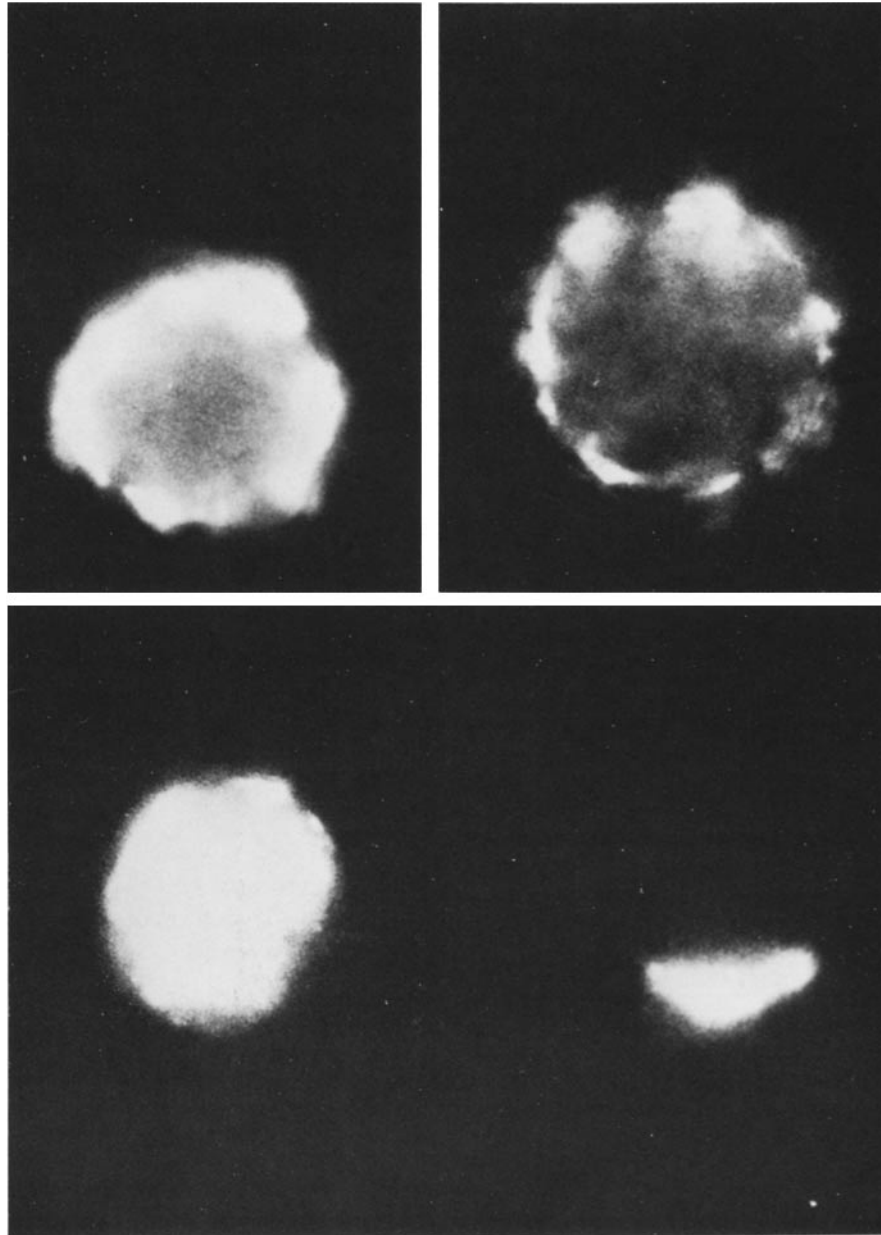


FIG. 8. Human C3 fluorescence microscopy of cells treated with antibody and NHS. Cells which were treated similarly except for substitution of CR for antibody did not show any deposition of human C3.  $\times 500$ .

suggested by the recent work of Unkeless et al. (36). In attempting to explain these stimulatory effects it seems likely that glycopeptides and glycoproteins are being removed from the cell surface resulting in secondary effects on membrane transport. In skeletal muscle and adipose tissue trypsin produces rapid and dramatic changes in glucose transport (37).

The possible relationship of our observations in tumor cells to the recently postulated role of C component C3 in B-cell activation needs to be considered. Dukor and Hartman have proposed that B-cell activation takes place by mitogen bridging of membrane receptors with concomitant interaction of activated C3 with the C3 membrane receptor (38). Theofilopoulos et al. have demonstrated binding sites for C3 and C3b on the human lymphoblastoid cell line (RAJI); the binding of C3b to the cell activated the alternate C pathway and led to membrane damage and cytolysis (39). While these studies suggest that C or C components play a crucial role in B-lymphocyte growth and function they do not pertain to the question of whether or not other cells which presumably lack well-defined receptors for C would undergo a similar response. Our studies with a mouse fibroblastic cell line suggest that the activation effects of C are part of a more general biological role of C in the promotion of cell growth during inflammation. It is well known that fibroblastic growth and proliferation are important in tissue responses to infection and trauma.

In addition to the collaborative effect of anti-L-cell antibody and C on nucleoside incorporation, DNA synthesis, and cell growth in these cells, there are less marked changes in these parameters in cells treated with antibody alone. Since we excluded C merely by heat inactivation, some heat-insensitive C components may be interacting with antiserum and cells to produce immunostimulation. However, since some of the C components needed for C-mediated stimulation of cell growth are heat labile and there is residual stimulation with component-deficient sera, this explanation is unlikely. In C-free systems antibodies have been reported to activate potassium (40) and glycine (41) transport so direct effects of antibody on plasma membrane function appear possible.

Even though C is apparently not required for a response, when it is present the minimal stimulatory concentration of antibody is reduced by several orders of magnitude. The studies with sera deficient in selected C components indicate that most of the effect of C, particularly that at low concentrations of antibody, is exerted through the classical C pathway. Thus C4D was a poor stimulant of cell growth even though it has alternate pathway activity. At high concentrations of antibody, however, it did potentiate the response. These findings are similar to those reported for sensitized sheep erythrocytes where the classical pathway is required for C activation of lightly sensitized erythrocytes and the alternate pathway can be activated when cells are heavily sensitized. This has been analyzed in detail and found to proceed via the activation of a calcium-dependent C1-like protein which in turn activates alternate pathway components (42, 43).

The data presented in this paper clearly show that C has a dual action with respect to target cell growth with stimulation at low antibody concentrations and inhibition at high antibody concentrations. While the relationship of these observations to the phenomenon of antibody enhancement of tumor cell growth *in vivo* remains to be conclusively established, we have recently shown that properly chosen doses of passively administered anti-L-cell antibodies significantly stimulate L-cell growth in antibody-treated C3H mice deprived of T-cell function (lethally initiated, thymectomized, and bone marrow reconstituted)

(44). Since the recipient animal's own immune response is already markedly impaired it seems likely that the antibody is acting directly on tumor cell metabolism rather than indirectly through specific inhibitory effects on the host's immune system. Further experiments using C-depleted animals are in progress and should help to define the role of C in the in vivo response.

### Summary

When L cells were treated with anti-L-cell antibody in medium containing heat-inactivated fetal calf serum, nucleoside uptake and cell growth were stimulated. The response was markedly increased when fresh, unheated sera from calves, guinea pigs, humans, mice, or rabbits were also present. The factors in unheated serum responsible for the enhancement of immunostimulation were studied. Using low concentrations of sera deficient in various complement (C) components and low concentrations of antibody no augmentation of immunostimulation was seen with C1r-deficient human serum, C2-deficient human serum, C2,4-deficient human serum, C4-deficient guinea pig serum, C3-C9-depleted guinea pig serum (by administration of cobra venom factor to animals), but stimulation was observed with C5-deficient human serum, C5-deficient mouse serum, and C6-deficient rabbit serum. When the concentration of anti-serum was raised, however, augmentation was observed with C4-deficient guinea pig serum. Thus, at low concentrations of antiserum enhancement appeared to occur through the classical C pathway, whereas at high concentrations of antibody either the classical or alternate C pathways appeared to be involved. Stimulation was specifically restored by purified C2 in C2-deficient serum and by C3 in C3-C9-deficient serum. Under the usual reaction conditions consumption of guinea pig C component C4 could be demonstrated which provided direct evidence for activation of the classical C pathway under conditions leading to immunostimulation. By immunofluorescence, cells treated with antibody and normal human serum had human C3 deposited at the cell surface.

Taken together these observations suggest that C activated through C3 by either the classical or alternate pathways has the potential to enhance nucleoside incorporation into DNA and cell growth of cells exposed to limiting amounts of antibody. Although the mechanism of stimulation is unknown, it is likely to involve a direct effect of C3 at the level of the cell membrane.

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