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THE MICROENVIRONMENT OF THE LYMPHOCYTE CELL SURFACE IN TUMOROUS AND NON-TUMOROUS HOSTS

FRANCIS PETER CUCCHIARA JR.

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THE MICROENVIRONMENT OF THE LYMPHOCYTE
CELL SURFACE IN TUMOROUS AND
NON-TUMOROUS HOSTS

by


Francis P. Cucchiara, Jr.
B.S., Eastern Nazarene College, 1969

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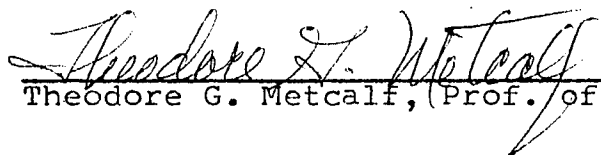
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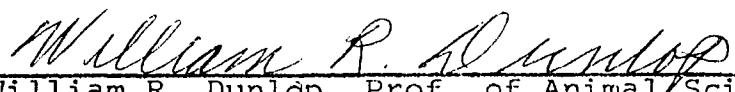
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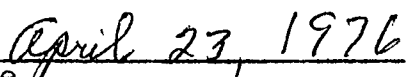
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ABSTRACT

THE MICROENVIRONMENT OF THE
LYMPHOCYTE CELL SURFACE
IN TUMOROUS AND NON-TUMOROUS HOSTS

by

FRANCIS P. CUCCHIARA, JR.

When peripheral and splenic lymphocytes from normal uninoculated chickens were stained with acridine orange and viewed with a fluorescent microscope, the cells produced a green intracellular fluorescence indicating that DNA was the only detectable nucleic acid within these cells. However, lymphocytes harvested from chickens manifesting RSV-induced progressing tumors produced two types of fluorescing cells. The first type appeared green and constituted approximately 80% of the observed cells. The second type produced an irregular red fluorescence at the cell periphery in addition to an intracellular green fluorescence.

Red fluorescing lymphocytes were evident in the peripheral blood of inoculated hosts 3 days post-inoculation. Parallel increases in cell numbers were noted in all infected animals for approximately the next 4 days after which further increases and/or decreases were related to tumor progression or regression.

Inoculation of chickens with three nononcogenic RNA viruses, or with bacterial antigen, did not produce similar patterns of red fluorescence indicating that the

staining technique might be specific in detecting viral oncogenesis.

When lymphocytes from hosts with progressing tumors were treated with RNase virtually all red fluorescence was eliminated but treatment with either DNase or trypsin had no effect. On the other hand, RNase treatment of lymphocytes harvested from hosts with regressing tumors did not affect the number of red fluorescing cells.

Washing lymphocytes from hosts with progressing tumors in phosphate buffered saline caused a significant reduction in the number of red fluorescing cells. Subjection of these washings to spectral analysis at a wavelength of 260 nm and to the orcinol test verified that RNA was being released from progressor lymphocytes. Washings of lymphocytes from tumorless chickens gave negative results for both tests.

Polyacrylamide disc gel electrophoresis of sera from uninoculated chickens, and hosts with progressing and regressing tumors, demonstrated that a low molecular weight protein was present only in sera of hosts with progressing tumors.

INTRODUCTION

The sensitized lymphocyte is the primary immunological mediator displaying surveillance and cytotoxic activity to the tumor cell. However, in most tumor systems under investigation, the immune lymphocyte is unable to express itself, and therefore incapable of arresting the multiplication of the neoplasia. Although the exact mechanism by which the sensitized lymphocyte is inactivated is still unresolved, existing data indicate that lymphocyte desensitization occurs on or near the cell surface.

Acridine orange, at pH 6, stains nucleic acids with two distinct fluorescence patterns. Therefore, in the present study, experimentation was undertaken to determine: 1) whether lymphocytes from tumor-bearing and non-tumor bearing (uninoculated) chickens could be differentiated on the basis of their fluorescence characteristics using the fluorochrome acridine orange and 2) whether or not a substance(s) was present on the surface of the lymphocyte of the neoplastic host but absent on lymphocytes from normal hosts.

REVIEW OF THE LITERATURE

THE ROLE OF THE SENSITIZED LYMPHOCYTE
IN TUMOR IMMUNOLOGY

The significant role that the T lymphocytes play in the surveillance and destruction of the tumor cell is well documented. In chickens (Hellstrom and Hellstrom, 1970; Sjogren and Jonsson, 1970), as in most tumor systems (Hellstrom and Hellstrom, 1967; Rouse and Warner, 1973; Gillette and Belante, 1973), lymphocytes from individuals that possess Rous sarcoma virus (RSV)-induced progressively growing sarcomas are unable to express their cell mediated immunity to transformed cells.

Hellstrom and Hellstrom (1969) initially attributed this loss of cellular activity to the coating of lymphocytes with antibody. Subsequently, Sjogren, Hellstrom, Bensal, and Hellstrom (1971) presented evidence which implicated antigen-antibody complexes as the coating material. This hypothesis is substantiated somewhat by the fact that although cytotoxic antibody was found in sera of tumor-bearing animals, antibody alone could not block lymphocytic activity until solubilized tumor-specific transplantation antigen (TSTA) was added to the system (Baldwin, Price and Robbins, 1972).

In contrast to this hypothesis, Currie and Basham (1972) implicated TSTA as the substance responsible for lymphocyte "desensitization". This view is also shared by

Smith (1974) who demonstrated the presence of TSTA in both the venous and lymphatic circulation in individuals with progressing tumors while upon removal or regression of such tumors, TSTA disappeared.

Although the exact mechanism by which lymphocytic activity is altered in the tumorous animal is not known, the data of Sjogren et. al (1971) and Currie and Basham (1972) demonstrated that a difference does exist between sensitized (responsive) lymphocytes and lymphocytes obtained from individuals possessing progressively growing tumors.

CELLULAR STAINING WITH ACRIDINE ORANGE

At the proper pH, acridine orange, an intercalating agent used as a fluorochrome, stains nuclear DNA with a green fluorescence and the nucleolus and cytoplasmic RNA with a red fluorescence. Proliferating malignant cells are readily characterized by an RNA content which greatly exceeds that of the non-malignant cells of origin (Bracket, 1950; von Beralanffy and Bertalanffy, 1960). Thus these changes in cytoplasmic constituents could readily be observed by acridine orange (A O) staining.

The method employed for cytodiagnosis of cancer utilizing acridine orange was first developed for exfoliative cytology in gynaecology and later applied to the diagnosis of malignancies of the respiratory system. This technique, based upon the metachromatic properties of acridine orange, involved differential staining between the

two types of nucleic acids of the cell (von Bertalanffy and Beckis, 1956).

Because of its potential importance as a diagnostic tool, acridine orange staining is extensively used in Canada and in the United States of America. In both countries, cancer diagnosis by exfoliative cytology had found increasing clinical application (Bertalanffy, 1960).

The study of normal, atypical and neoplastic blood corpuscles of the red and white series by means of fluorescence microscopy is of considerable interest both practically and theoretically.

Although the blood has been extensively investigated by a variety of methods and techniques, the field of blood pathology still possesses numerous limitations, especially in the areas of cytology and morphology. The difficulty arises in the interpretation of morphologically similar blood cells by the classical techniques. Now the various techniques of fluorochroming offer the possibility of intravital staining as well as observation of fixed material both of which are undoubtedly useful as illustrated by the existing literature on the subject (Eder, 1958; Jackson, 1961; Pimenta, 1960; Schiffer, 1962).

ELECTROPHORETIC TECHNIQUES IN THE ANALYSIS OF CHICKEN SERUM PROTEINS

Electrophoretic studies on chicken serum have been reported using various techniques. Five distinct peaks

were observed by moving-boundary electrophoresis (Moore, 1948); 5-6 bands were demonstrated by paper electrophoresis (Common and McKinley, 1953); and 6-7 bands were identified using cellulose acetate electrophoresis (Longenecker, 1967; Beg and Clarkson, 1970). Incorporating the molecular sieving concept 12 bands were found by starch-gel electrophoresis (Amin, 1961; Ogden, 1962) and 10-13 bands were distinguished using polyacrylamide-gel electrophoresis (Glick, 1968; Harris and Sweeney, 1969).

Medina, Rhodes and Mussman (1971) studied normal chicken serum by polyacrylamide-gel electrophoresis and found 13 bands. Since the nature of each protein band has not been established, only those for which identity is known are named, using the nomenclature of Glick (1968) and Prosky (1969). Prior to 1970, separation of protein on polyacrylamide-gel electrophoresis depended not only on the charge of the proteins but also on molecular size. Therefore, it is extremely difficult to make a direct comparison of this technique with the results obtained from cellulose acetate or paper electrophoresis.

MATERIALS AND METHODS

HOST AND VIRUS

A highly inbred line of White Leghorn chickens (Line 6₃), which is resistant to MDV (Crittenden, Muhm and Burmester, 1972) and GS antigen positive (Crittenden, Wendell and Motta, 1973) was obtained from H. A. Stone of the Regional Poultry Research Laboratory, East Lansing, Michigan and used as host unless designated otherwise. A highly purified preparation of a RSV pseudotype, the Bryan high titer strain, was obtained from Dr. L. B. Crittenden, Avian Physiology Laboratory in Beltsville, Maryland. The left wing-web of chickens either 3 days or 6 weeks of age was inoculated subcutaneously with 0.1 ml of a 10⁻³ dilution of stock virus containing 2,000 pock-forming units per ml. Where splenic lymphocytes were employed, uninoculated (normal) chickens and chickens displaying progressively growing tumors (progressors) or regressing tumors (regressors) were sacrificed usually between 21 and 40 days of age and the spleens aseptically removed.

SPLENIC LYMPHOCYTE PREPARATION

Splenic lymphocytes from progressor, regressor or normal chickens were harvested by differential centrifugation as follows: all connective tissue surrounding the outer surface of the spleen was removed and the organ thoroughly minced in a tube containing phosphate-buffered saline

(PBS) (Dulbecco and Vogt, 1954). The supernatant fluid was carefully removed and centrifuged at 600 x g for one minute. The supernatant liquid was then subjected to centrifugation at 800 x g for one min at 22 C followed by a final centrifugation at 1400 x g for 15 min at 4 C. The liquid was then discarded to within 10 mm of the pellet and the band of lymphocytes (white in appearance) was gently removed from the top of the pellet. The lymphocyte harvest which represented from 37% - 57% of the initial population was suspended in fresh PBS. In most instances, lymphocytes were stained within 2 hours. However, storage of cells for 48 hours before staining did not result in changes in cell number or surface staining characteristics.

PERIPHERAL LYMPHOCYTE PREPARATION

Peripheral lymphocytes were obtained by employing a Ficoll-Hypaque mixture (Archambault, Dunlop, Cucchiara, Collins and Corbett, 1976). Solution of 9% Ficoll (Sigma Chemical Co., St. Louis) and 33.3% Hypaque (Winthrop Laboratories, N.Y., N.Y.) were mixed in 24:15 proportions respectively and sterilized by autoclaving at 110 C for 30 min (specific gravity, 1.090). Four ml of mixture was added to 15 ml conical centrifuge tubes and 2 ml of heparinized blood (obtained from the wing vein) gently layered onto the Ficoll-Hypaque mixture. Each preparation was centrifuged at 800 x g for 3.5 min at 4 C in a Sorvall HS-34 rotor and the white cell layer above the Ficoll-Hypaque mixture removed with a

capillary pipette. This technique produced lymphocyte recoveries of 90% or greater.

ACRIDINE ORANGE STAINING

Staining of lymphocytes with acridine orange (Fischer Scientific Company, Medford, Mass.) was as described by Gurr (1962). Lymphocytes were fixed wet for 5-15 min in a 1:1 solution (by volume) of 95% ethyl alcohol and anhydrous ether. The fixed preparations were then subjected to graded alcohol (80, 70 and 50% ethyl alcohol) rinses for 10 seconds and rinsed for 10 seconds in distilled water. The specimens were then immersed into a solution of 1% glacial acetic acid for one min to prevent rapid fading of fluorescence. The slide was rinsed in distilled water and stained in a buffered 0.02% acridine orange solution for approximately 3-5 min. The staining solution consisted of the following ingredients. Stock phosphate buffers were prepared by using 0.072 g/l and 9.465 g/l of KH_2PO_4 and Na_2HPO_4 respectively in deionized distilled water (pH 7.0). The working phosphate buffer solution contained 300 ml of KH_2PO_4 , and 50 ml of Na_2HPO_4 , adjusted to pH 6. Two grams of acridine orange were dissolved in 100 ml of deionized distilled water and 0.5 ml added to 99.5 ml of phosphate buffer. Fresh stain was prepared for use in all experiments. Once stained, the excessive dye was removed by immersing the slide lymphocyte preparation into phosphate buffer for one minute.

To promote differentiation between RNA and DNA, the stained preparation was placed in 0.1M CaCl₂ for 2 min. Once differentiated, the stained sample was rinsed in phosphate buffer for one min for immediate viewing or left in the buffer until viewed under the Reichert fluorescent microscope (exciting filter no KG-2/BG-12, and barrier filter no. 1.5/OG1-1/BG9).

SDS-POLYACRYLAMIDE DISC-GEL ELECTROPHORESIS

Disc-gel electrophoresis was performed on all samples using the Model 1200 apparatus manufactured by Canalco Corporation, Rockville, Maryland. The procedure was as recommended by Laemmli (1970) except for the following modifications: (1) the concentration of acrylamide in the separating gel was 7%; (2) the samples were not heated; (3) the column coat was omitted; and (4) the gels were destained in 7% acetic acid employing the Model 1801 Quick Destainer (Canalco Corporation, Rockville, Maryland). Gels were evaluated from tracings made with the Joyce-Loebl Chromoscan (Burlington, Mass.) with integrator. The protein concentration TSTA, serum and washing samples was determined as described by Lowery (1951). Protein ranging in concentration from 200-600 ug was loaded onto appropriate gels and triplicate samples were analyzed simultaneously.

Blood samples (4 ml) were obtained by cardiac puncture and collected in sterile 10 ml screw cap tubes. The blood samples were allowed to clot for 30 min at 37 C.

The serum was then transferred by capillary pipette to a sterile 10 ml screw cap test tube and was stored at 5C.

RESULTS

PROPERTIES OF THE LYMPHOCYTE CELL SURFACE

When lymphocytes extracted from the spleen of a 6-week old uninoculated chicken were treated with acridine orange as described in Materials and Methods, the cells produced (almost without exception) a green intracellular fluorescence indicative of DNA (Figure 1). From a total of 627 cells counted only 2 demonstrated red fluorescence. On the other hand, the lymphocytes of a 3-week old chicken manifesting a maximum wing-web tumor produced two types of fluorescing cells. The first type demonstrated a green fluorescence similar to that observed in the control chicken and comprised approximately 77% of the total cell population. The second cell type possessed an intracellular green fluorescence and an irregular red fluorescence at the periphery (Figure 2). These initial observations indicated that acridine orange could be used for differentiating lymphocytes from normal vs. tumor-bearing chickens and suggested that the material producing the red fluorescence at the cell periphery was RNA.

Because the lymphocytes in the foregoing experiments were obtained from hosts of different ages, two additional splenic cell samples were obtained from normal and progressor chickens and subjected to acridine staining. The combined results (Table 1) indicate that age was not the factor responsible for the red fluorescence of progressor lymphocytes.

Figure 1. Photomicrograph of lymphocytes
harvested from a host showing
no signs of tumor. (1900 x)

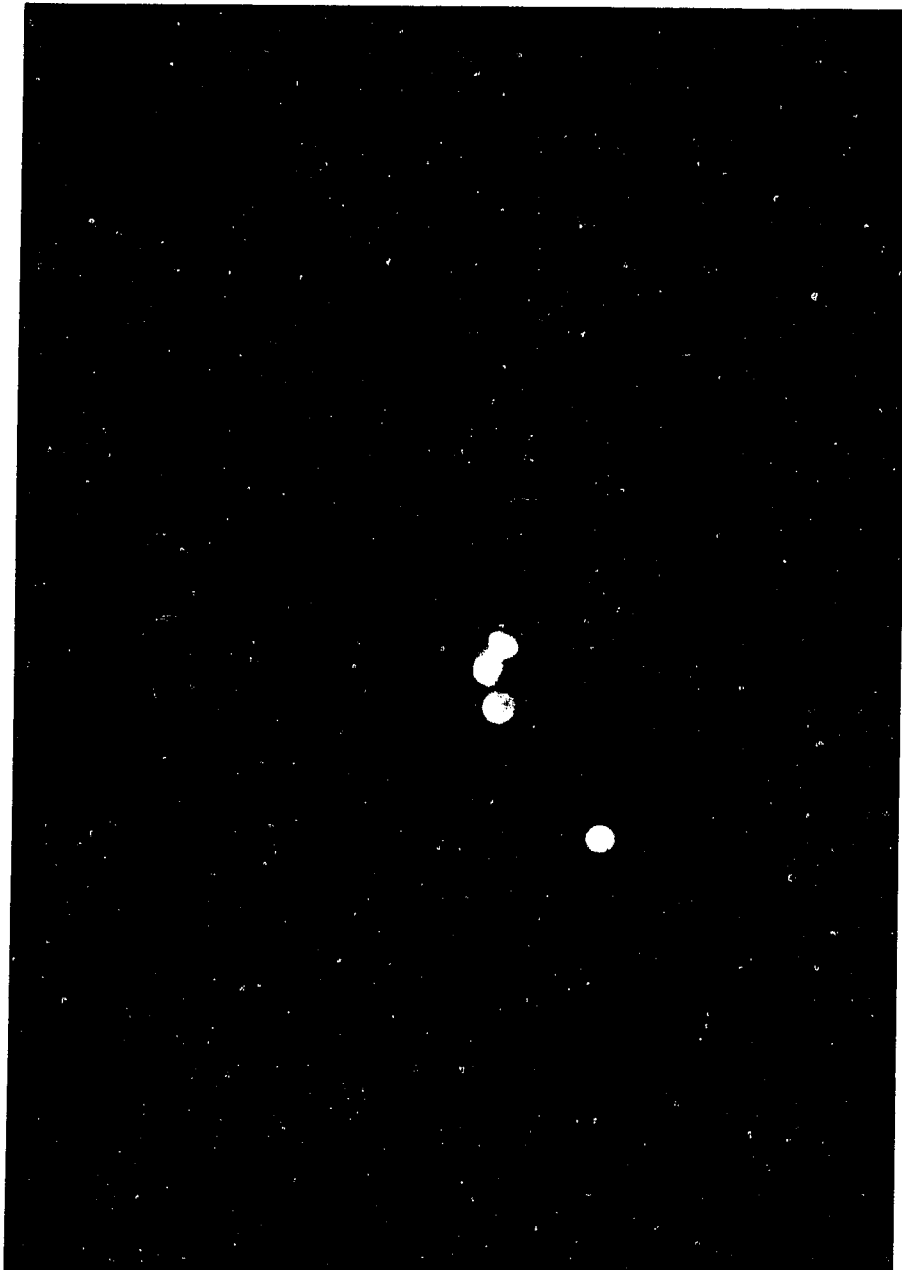


Figure 2. Photomicrograph of lymphocytes
harvested from a host with
progressing tumor. (1900 x)

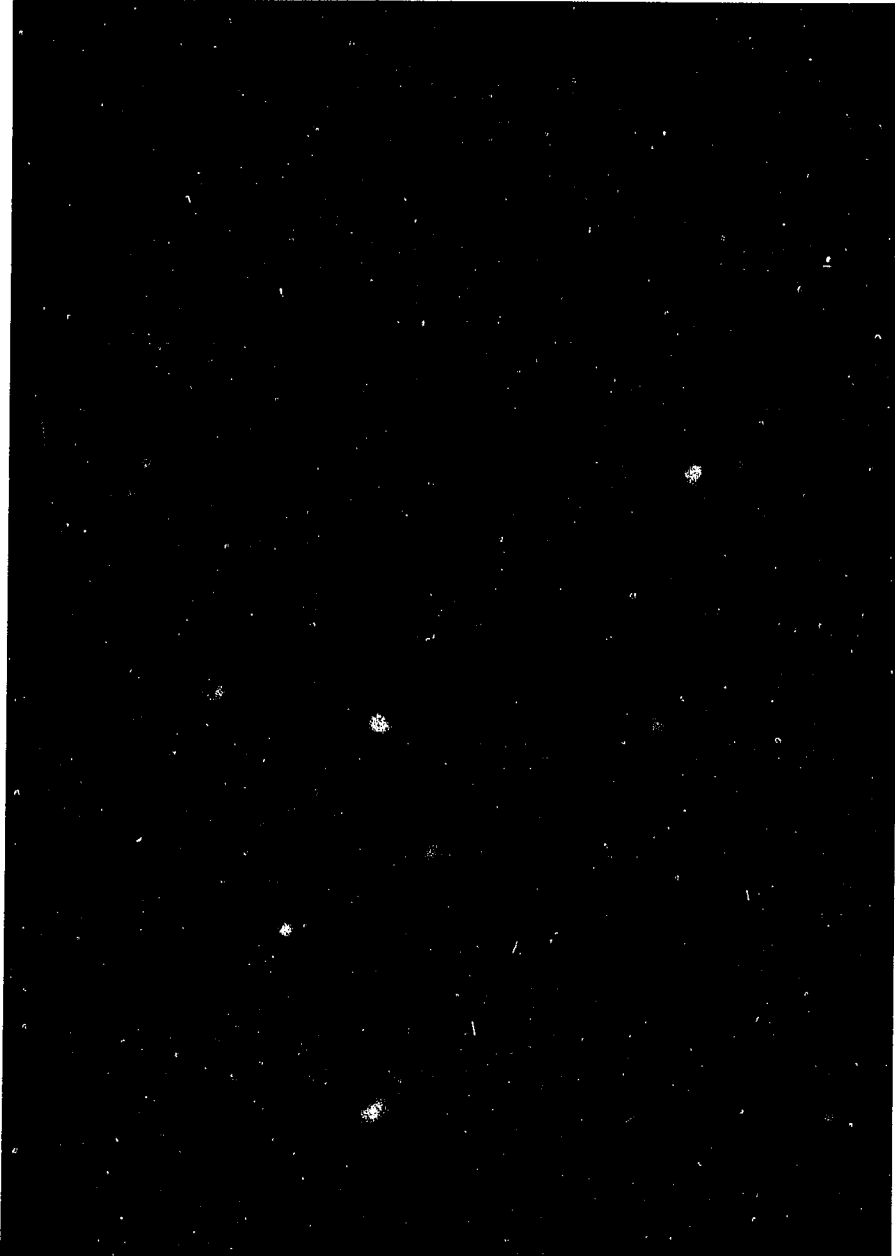


Table 1

Fluorescence characteristics of splenic lymphocytes harvested from non-tumor bearing and tumor-bearing hosts following acridine orange staining

Type of host	Experiment No.	Age of host (weeks)	Lymphocytes Fluorescing (no.)		Green Fluorescing lymphocytes (%)
			green	green & red	
Control (non-tumor-bearing)	1	6	625	2	99.7
	2	36	586	1	99.8
	3	4	531	2	99.6
Progressor	1	3	513	147	77.7
	2	4	505	144	77.8
	3	4	536	152	77.9

SPECIFICITY OF A.O. STAINING

Fluorescence characteristics of splenic lymphocytes from groups of chickens inoculated at 1 week of age with either oncogenic or a nononcogenic virus are compared in Table 2. Stained lymphocytes from spleens of animals inoculated with RSV produced an increase in red fluorescing cells beyond those observed in spleens of chickens inoculated with nutrient broth. Lymphocytes from animals inoculated with a nononcogenic RNA virus, regardless of type, showed no increase in red fluorescence over that observed in the negative control. On necropsy, each host injected with nononcogenic RNA virus possessed characteristic histopathological lesions as determined by sections of appropriate tissue. These results demonstrated what appeared to be a specificity of acridine orange staining in RSV-induced oncogenesis.

Fluorescence characteristics of peripheral and splenic lymphocytes from a group of chickens inoculated at 6 1/2 weeks of age with Salmonella pullorum O antigen were also studied. Two chickens were inoculated intravenously with 0.75 ml of a 9×10^8 organisms/ml (McFarland Standard No. 3 [McFarland, 1907]) of prepared Salmonella pullorum O antigen (Campbell, Garvey, Cremer and Susdorf, 1970). A control host was similarly inoculated with 0.70 ml of nutrient broth. All hosts were bled for sera and peripheral lymphocytes and sacrificed for spleen fifteen days post-inoculation. Lymphocytes from animals inoculated with

Table 2

Fluorescence characteristics of lymphocytes obtained from Spafas chickens infected with avian RNA viruses^a

Inoculum	Route of Infection	Dose ^b (ml)	Disease Symptoms	Lymphocytes fluorescing (no.)	
				green	green & red
Nutrient Broth	subcutaneous	0.1(bleb)	none	561	2
BH RSV (RAV 1)	subcutaneous	0.1(bleb)	present	561	166
Avian Encephalomyelitis Virus	oral	0.2	present	538	1
Infectious Bronchitis Virus	nasal	0.2	present	571	1
Newcastle Disease Virus	nasal	0.2	present	573	1

^aGroups of 7 chickens (1 week old) were injected with nutrient broth and the listed Avian RNA viruses. When symptoms of disease appeared, lymphocytes were harvested from spleens and the cells stained with acridine orange as described under Materials and Methods.

^bTiters of virus stocks, as determined by dilution end point, were 10^{-3} , 10^{-7} , 10^{-3} and 6.5 for RSV, AE, IBV and NDV respectively.

the bacterial antigen and nutrient broth showed no increase in red fluorescence over that observed in the negative control. Serum samples of all test subjects were analyzed for anti-Salmonella pullorum O antibody. A simple agglutination test demonstrated an antibody titer of 640 in those hosts inoculated with Salmonella pullorum O antigen. No detectable antibody titer appeared in the serum of the control. Again, these results further demonstrate a specificity of acridine orange staining in RSV-induced oncogenesis.

APPEARANCE OF RED FLUORESCING LYMPHOCYTES
IN INOCULATED HOSTS

An extended study was initiated to determine the time post-inoculation (with RSV) when red fluorescence initially appeared on the surface of the peripheral lymphocyte. Fourteen line 6₃ chickens inoculated at 6 weeks of age were studied and the average percentages of red fluorescing cells determined (Figure 3). Beginning on day 3 post-inoculation and continuing to approximately day 10, a rapid rise in red fluorescing cells was noted. Thereafter, further increases in cell numbers were observed until day 20, when the percentage of red fluorescing cells began to decline. In approximately 90% of the injected hosts, tumors were not visible until day 11 (Table 3).

These data are seen in different perspective when the lymphocyte response of each host is plotted separately (Figures 4-8). In progressor hosts with maximum (Cotter,

Figure 3. Average percentages of red fluorescing lymphocytes appearing in the peripheral blood of 14 RSV-inoculated hosts (▲----▲) and 3 uninoculated controls (O-----O).

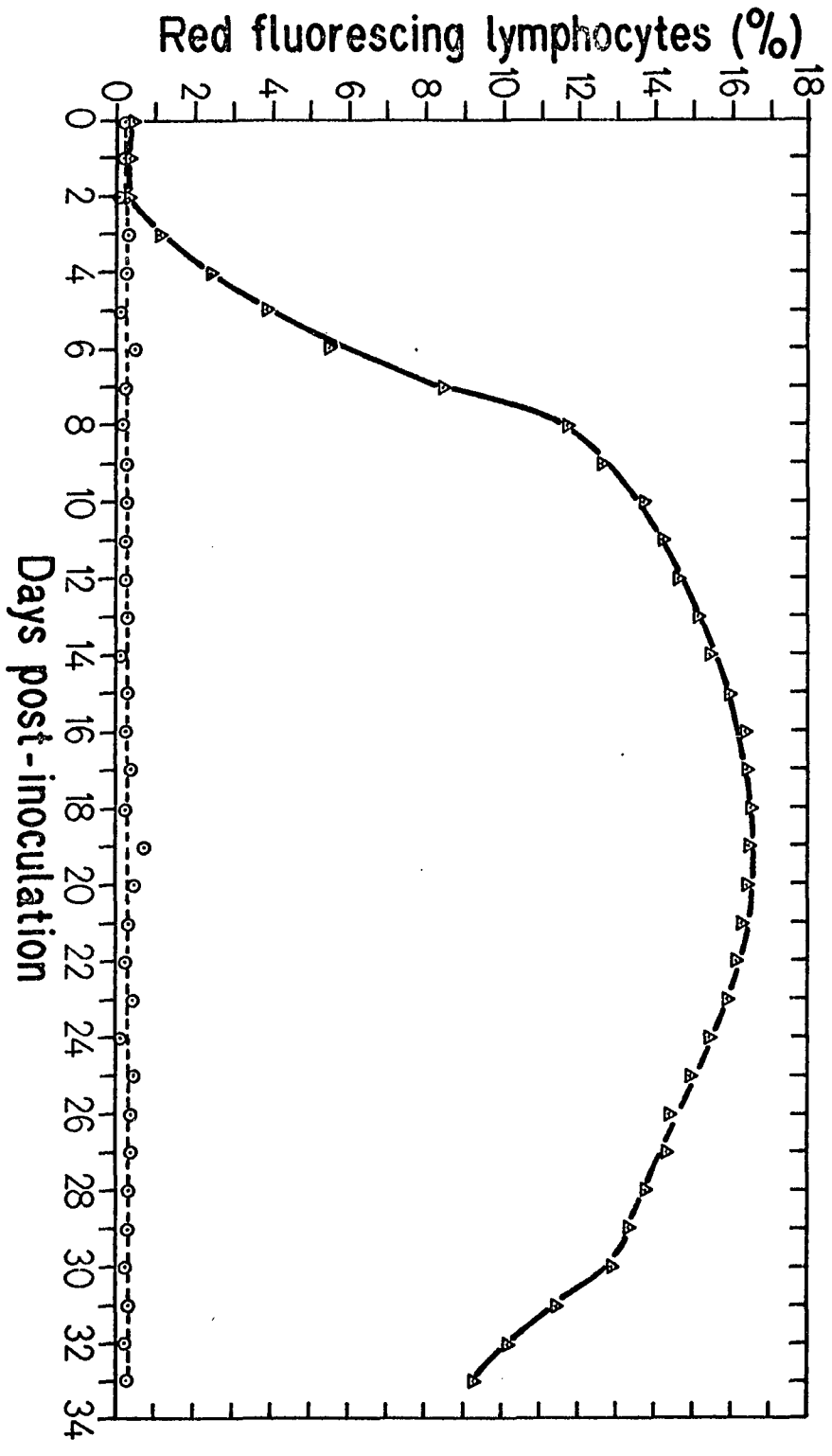


Table 3

Tumor scores on Line 6₃ hosts inoculated with RSV

Host	Subjective tumor score ^a on day- (post inoculation)																								
	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1555	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1559	0	0	1	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	0
1560	0	1	2	3	4	4	4	4	4	4	4	4	3	3	1	1	1	1	1	1	1	1	0	0	0
1565	0	0	1	2	3	3	3	4	4	4	4	4	4	3	3	2	2	1	1	1	1	1	1	0	0
1573	0	0	1	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	0	0	0	0	**	**
1586	0	0	1	1	3	3	3	3	3	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1596	0	0	1	2	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	*	*
1598	0	1	2	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	*
1600	0	0	1	2	3	3	3	3	3	3	2	2	1	1	1	1	1	1	1	1	0	0	0	0	0
1601	0	0	1	1	2	2	2	2	2	2	1	1	1	1	1	1	1	1	2	2	2	1	1	1	1
1602	0	0	1	2	3	3	3	4	4	4	4	4	4	4	3	2	2	2	1	1	1	0	0	0	0
1603	0	0	1	2	2	2	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	*	*	*
1606	0	0	1	2	3	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	*	*
1607	0	0	1	2	2	2	3	3	3	3	3	2	2	1	1	0	0	0	0	0	0	0	0	**	**

^aTumor scores as described by Cotter et al. (3).

* Terminal

** Sacrificed for splenic lymphocytes.

Figure 4. Percentages of red fluorescing peripheral lymphocytes associated with 4 hosts that developed maximal (4) tumors and became terminal.

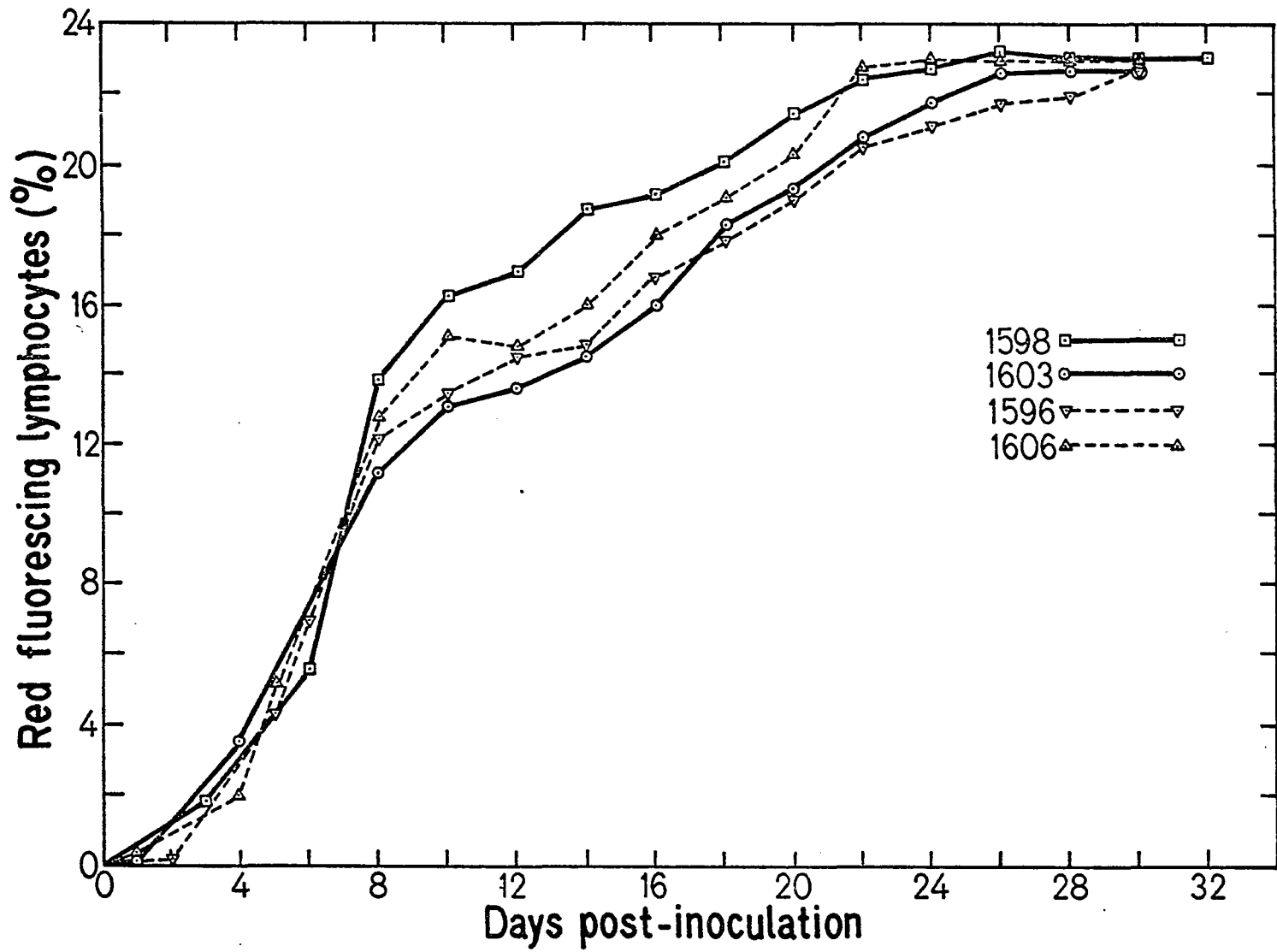


Figure 5. Percentages of red fluorescing peripheral lymphocytes associated with 3 hosts that developed maximal (4) tumors but ultimately regressed.

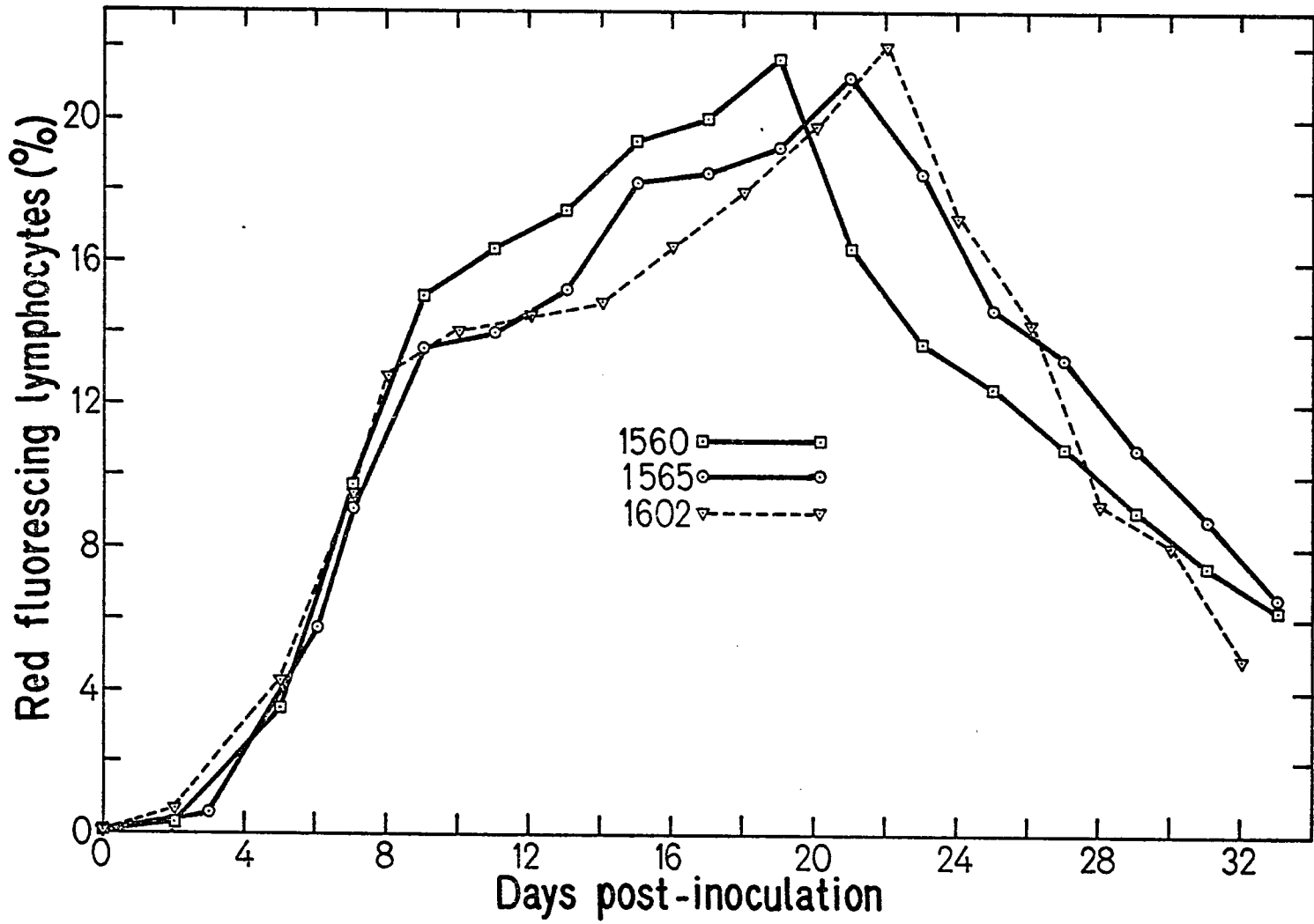


Figure 6. Percentages of red fluorescing peripheral lymphocytes associated with 3 hosts that developed a tumor score of 3, followed by regression.

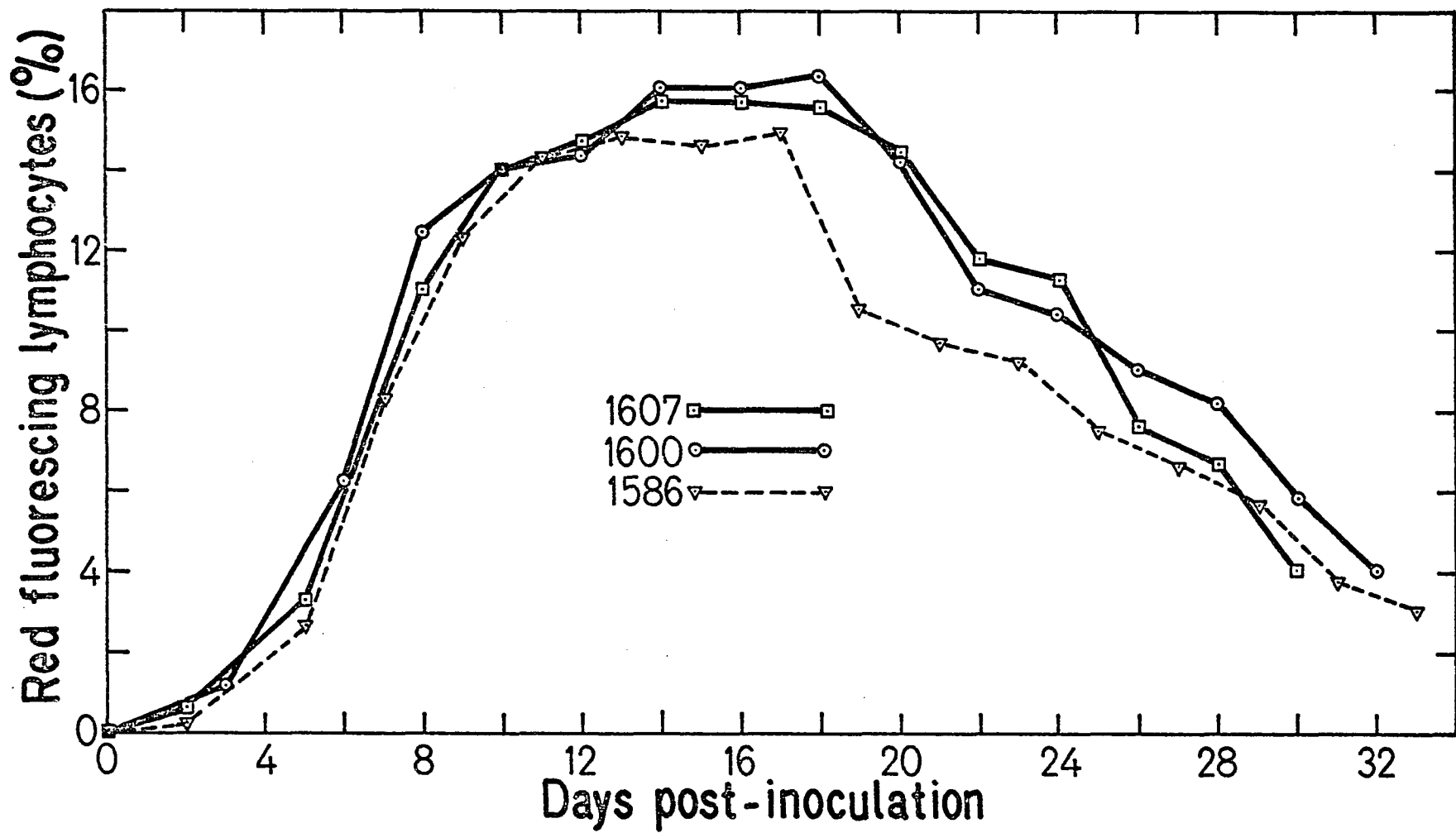


Figure 7. Percentages of red fluorescing peripheral lymphocytes associated with 3 hosts that developed a tumor score of 2, followed by regression.

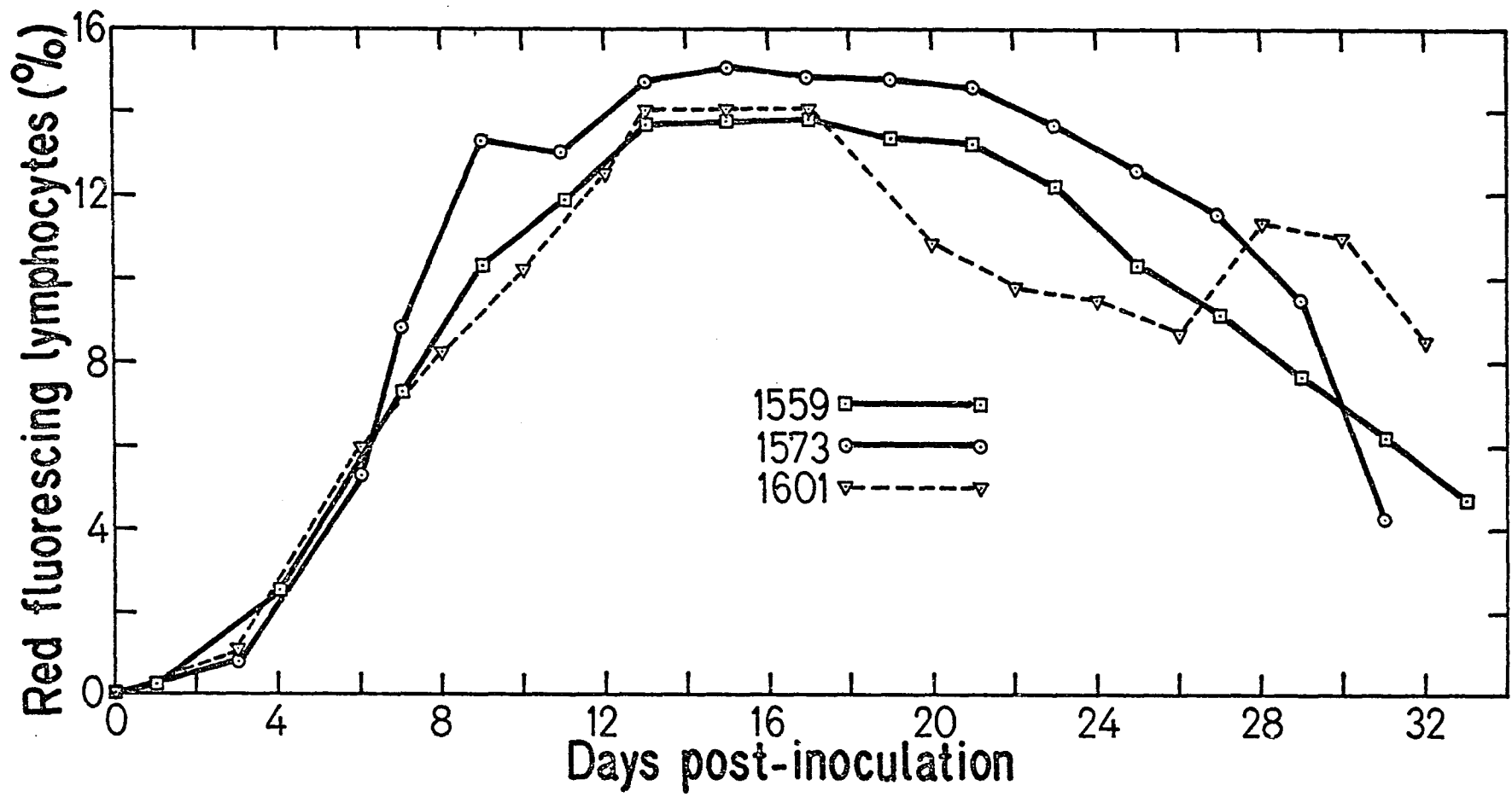
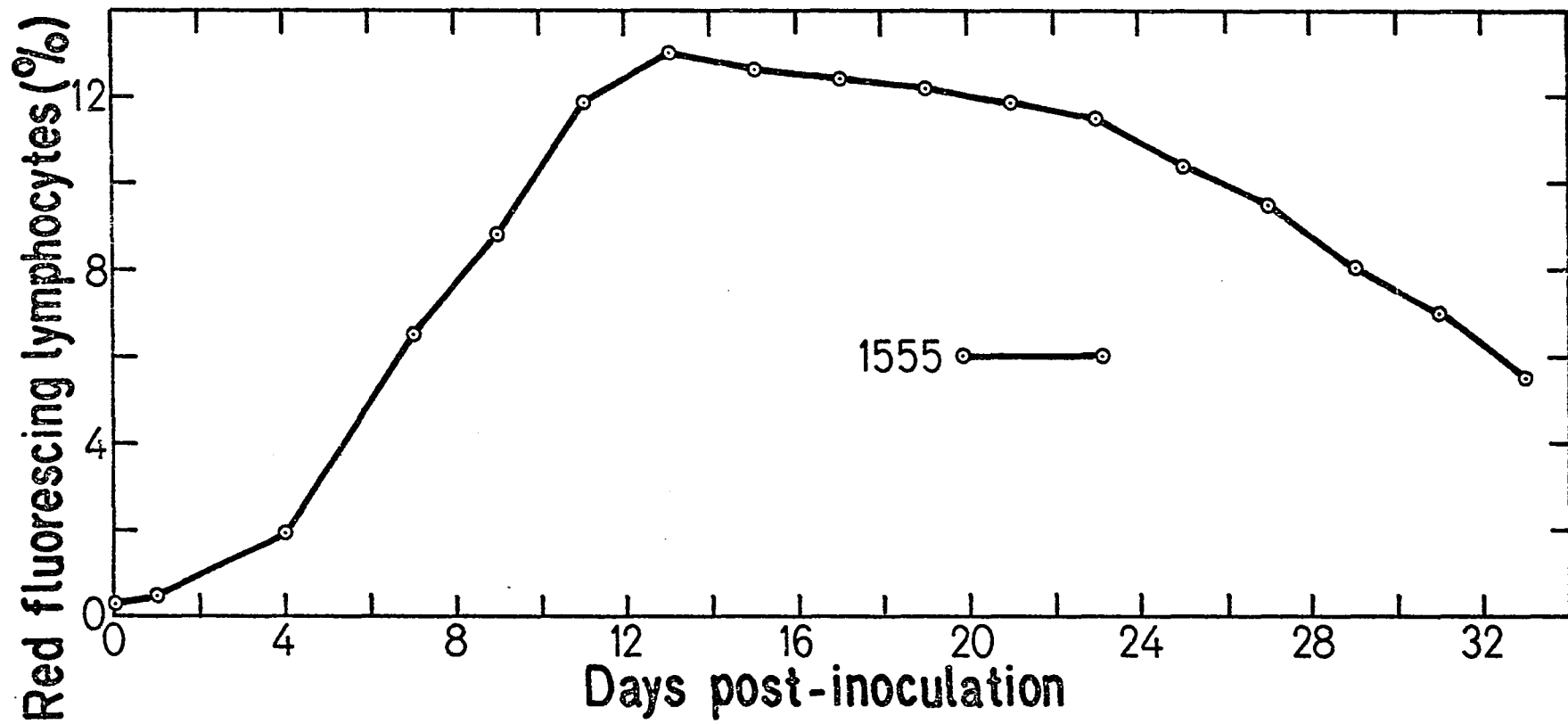


Figure 8. Percentages of red fluorescing peripheral lymphocytes associated with a host that developed a tumor score of 1, followed by regression.



Collins, Dunlop and Corbett, 1973) tumors (Figure 4), the percentage of red fluorescing cells increased steadily until it equaled that observed in earlier splenic preparation (Table 1). Three of the hosts became terminal on day 31, the fourth on day 32.

Likewise, Figures 5-8 represent lymphocyte response curves of regressor hosts afflicted with tumors of various sizes. In general, these data showed that the percentages of red fluorescing peripheral lymphocytes were maximum at approximately day 18 and that the higher percentages of red fluorescing lymphocytes were obtained from chickens possessing the larger tumor scores.

In 9 of the 10 hosts with regressing tumors, a decline in red fluorescing cells occurred 1 to 2 days prior to any observable decrease in tumor score (Table 4). The lymphocyte response curve of host 1601 is of special interest. Although an isolated observation in this experiment, the percentage of red fluorescing cells in this host increased between day 26 and day 28 followed by a corresponding increase in tumor size on day 27 (Figure 7 and Table 3). The validity of this observation was confirmed in a second study.

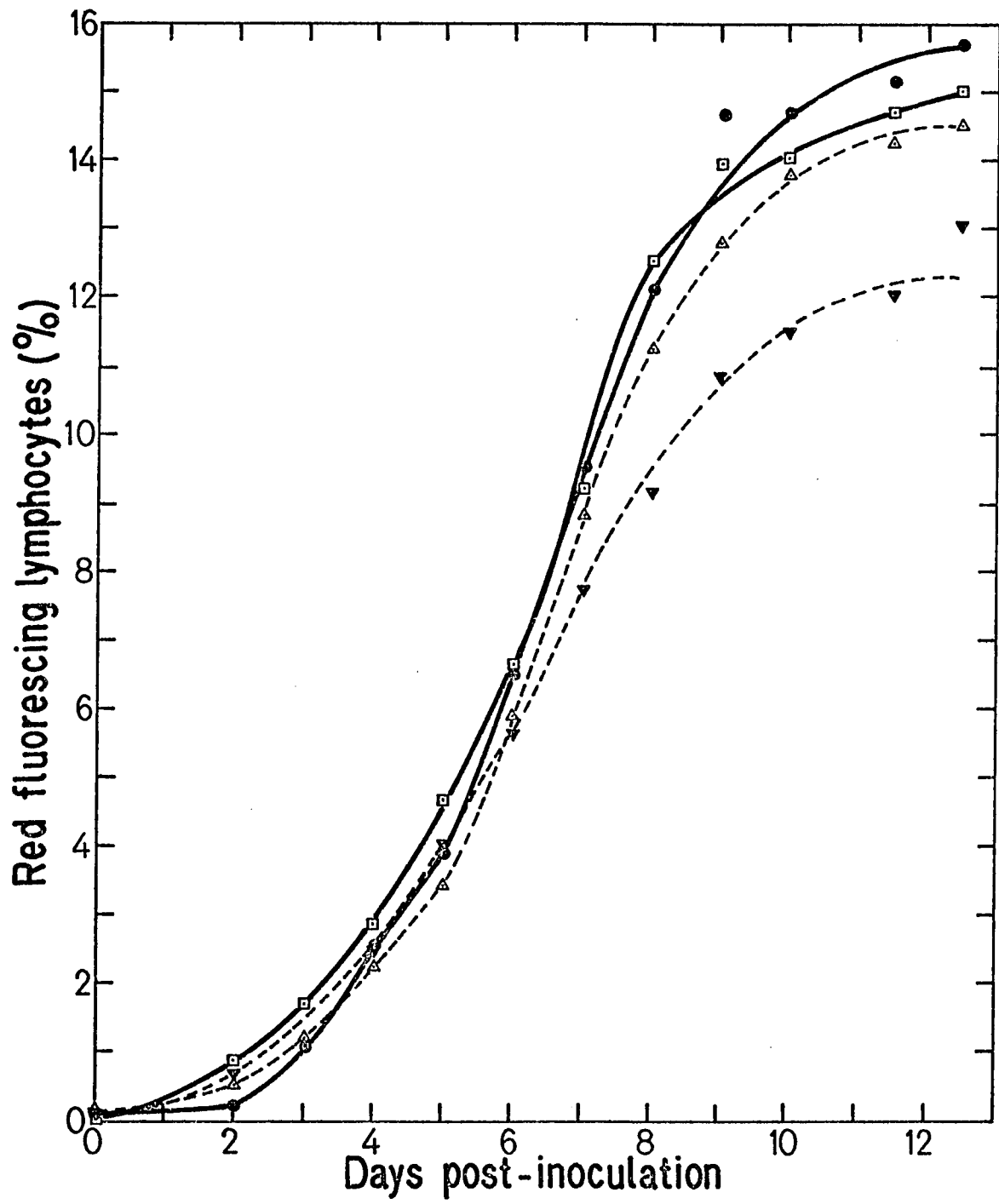
A comparison of the initial rise in red fluorescing cells in each group of infected hosts (Figure 9) suggested that the events occurring early in inoculated chickens were similar in all hosts during the first 7 days post-in-

Table 4

Relationship between decline in percentage red fluorescing lymphocytes and decrease in tumor score

Host	Maximum tumor score	Day post-inoculation when decrease first noted	
		Red fluorescing cells	Tumor score
1555	1	23	33
1559	2	21	23
1560	4	19	21
1565	4	21	22
1573	2	21	22
1586	3	17	18
1600	3	18	19
1601	2	17	19
1602	4	22	23
1607	3	18	19

Figure 9. Comparison of the initial rise in red fluorescing peripheral lymphocytes in hosts possessing various tumor scores. Average percentages calculated from Figures 4-7, []-----[], tumor score 4, progressor; ●-----●, tumor score 4, regressor; ▲ - - - ▲, tumor score 3, regressor; ▼ - - - ▼, tumor score 2, regressor.



oculation, regardless of eventual tumor size. Because of limited data regressor host 1555 (Figure 8) was not included in this analysis.

The results of a duplicate study performed over a 70 day period and involving 30 line 6₃ chickens, inoculated at 6 weeks of age, paralleled for the first 30 days those reported above. However of note was the data obtained from one of the inoculated (RSV) hosts. Although inoculated with virus, the host did not display any signs of tumor nor did acridine orange staining of peripheral lymphocyte show any increase in red fluorescing cells over the uninoculated control.

LOSS OF RED FLUORESCENCE BY RIBONUCLEASE TREATMENT

Experiments were conducted to determine if the material producing the red fluorescence at the periphery of the lymphocyte was indeed RNA. Harvested splenic lymphocytes from three progressors and three regressors were divided and one portion of the cells treated with RNase (50ug/ml final concentration; Worthington Biochemicals, Freehold, New Jersey) at 37 C for 30 min prior to fixing and staining. The second sample served as control. Nuclease-treated progressor lymphocytes (Table 5) demonstrated a green fluorescence similar to that observed in normal chickens. The enzyme reduced the percentage of red fluorescing cells from approximately 23.0% to less than 0.5%. A corresponding increase in green fluorescing cells was noted since such cells rose from approximately 77.7% to 99.6%. The agreement in

Table 5

Effect of RNase on removal of red fluorescing material
from progressor lymphocytes

Host no.	Lymphocyte treatment ^a	Number		Green fluorescing lymphocytes (%)
		lymphocyte fluorescing Green	Green & Red	
1	-	436	129	77.2
	+	569	2	99.6
2	-	453	130	77.7
	+	605	1	99.8
3	-	500	140	78.1
	+	644	3	99.5

^a - = without RNase.

+ = with RNase.

these values suggested that progressor lymphocytes could be "converted" to normal appearing lymphocytes by RNase. In a t-test of the difference between mean counts of red fluorescing cells in the untreated progressors vs. RNase-treated progressor lymphocytes, the t-value, 8.3 \leq (P 0.01) showed that the difference between these means was real.

On the other hand, RNase treatment of lymphocytes from regressor hosts did not influence the percentage of red fluorescing cells (Table 6).

The specificity of this enzymatic reaction in progressor lymphocytes was established by treating progressor lymphocytes with either DNase, trypsin or RNase (50 ug/ml final concentration of each, Worthington Biochemicals, Freehold, New Jersey) at 37 C for 30 min. A loss of red fluorescence occurred only in samples containing the added RNase (Table 7). In the DNase and trypsin-treated samples, 76.5% of the lymphocytes produced a green fluorescence; thus no distinction could be made between these data and those obtained in the untreated (progressor) control.

LOSS OF RED FLUORESCENCE BY WASHING

Currie and Basham (1972) reported that lymphocyte activity to tumor cells could be enhanced by washing the cells several times by centrifugation, enhancement being attributed to the removal of TSTA from the lymphocyte surface. This suggested that washing by centrifugation might result in the release of polynucleotides from the cell sur-

Table 6

Fluorescence characteristics of RNase-treated regressor lymphocytes

Host no.	Lymphocyte treatment ^a	Number lymphocytes fluorescing		Green fluorescing lymphocytes (%)
		Green	Green & Red	
1	-	520	17	96.8
	+	515	18	96.6
2	-	510	18	96.6
	+	507	19	96.4
3	-	633	23	96.4
	+	640	25	96.1

^a - = without RNase.

+ = with RNase.

Table 7

Enzymatic specificity involved in the removal of red fluorescence
from progressor lymphocytes^a

Enzymatic treatment	Number lymphocytes fluorescing		Green fluorescing lymphocytes (%)
	Green	Green & Red	
None	500	140	78.1
Trypsin	501	149	77.1
DNase	484	152	76.1
RNase	644	3	99.5

^aPortions of progressor lymphocytes from host no. 3 (Table 5) were subjected to trypsin and DNase as well as RNase.

face with a corresponding loss in red fluorescing cells. Consequently, harvested splenic lymphocytes were suspended in 10 ml PBS and the suspension centrifuged at 1400 x g for 15 min at 4 C. The supernatant was removed to within 5 mm of the lymphocyte pellet and transferred to a sterile test tube. Following resuspension of lymphocytes in 10 ml PBS, the washing process was repeated until a total of four separate washings were obtained. All washings were retained and stored at 5 C.

Staining the lymphocytes subsequent to washing resulted in a decrease in red fluorescing cells from approximately 23.0% to approximately 5.0%, indicating that washing effectively removed RNA from the cells. To verify this loss, each pool of washings was subjected to spectrophotometric analysis at 260 nm in a Bausch and Lomb Spectronic 600 employing PBS as blank. Substantial increases in adsorbance were noted in the initial washings of progressors #1 and #2 (Table 8). To eliminate the possibility that washing unselectively removed RNA from the lymphocytes, harvested lymphocytes from normal chickens were washed as described. No detectable increase in adsorbance at 260 nm was noted.

Additional verification was obtained by subjecting the first washing of lymphocytes from each host to orcinol reagent (Clark, 1964) employing purified RNA (Worthington Biochemicals, Freehold, New Jersey) prepared in PBS as standard. Ribonucleic acid was detected only in progressor samples, the total amount of orcinol reacting-substance being 10 and 25 ug/ml for host #1 and host #2 respectively.

Table 8

A₂₆₀ values of material released from progressor lymphocytes^a

Source of lymphocytes	Adsorbance at 260nm (Wash sample no.)			
	1	2	3	4
Normal control	0.004	0.003	0.000	0.000
Progressor #1	0.210	0.055	0.025	0.020
Progressor #2	0.660	0.410	0.060	0.030

^a Normal and progressor lymphocytes were washed four times with 10 ml PBS and a 3 ml sample of each of the washings subjected to spectrophotometric analysis at 260 nm.

Thus, removal of RNA from the progressor lymphocyte was not unselective but was specifically associated with the lymphocytes of tumor-bearing chickens.

EVIDENCE FOR RNA-PROTEIN COMPLEX

An experiment was performed to determine whether the RNA isolated from the surface of the progressor lymphocyte was complexed with protein. Lymphocyte washings obtained from progressor hosts were treated with an equal volume of isoamyl alcohol-chloroform (24:1 v/v) mixture, centrifuged at 1400 x g for 15 min and the aqueous phase subjected to adsorbance at 260 nm. An adsorbance of 0.15 was obtained. A duplicate sample was initially treated with 1% sodium lauryl sulphate (SDS) solution at 60 C for 15 min and then processed as the previous sample. An adsorbance of 0.33 was obtained, thereby illustrating that the SDS treatment apparently dissociated the protein from RNA with a resulting increase in 260 adsorbing material.

SDS-POLYACRYLAMIDE DISC-GEL ELECTROPHORESIS

Polyacrylamide disc-gel electrophoresis of sera from 7 normal, 6 regressors and 7 progressor hosts was performed to determine whether or not differences existed in the serum components of these hosts. At 6 1/2 weeks of age, male and female, immunologically competent hosts were bled for sera and the protein concentrations of each determined as described by Lowery (1951). Approximately 200 to 600 ug

of protein were analyzed by SDS disc-gel electrophoresis (Laemmli, 1970).

Densitometer analysis of the gels indicated the presence of approximately 17 bands from normal and regressor sera (Figures 10 & 12). On the other hand, 18 bands were evident in samples of progressor hosts (Figure 11). No distinction in the banding patterns could be seen between male and female sera.

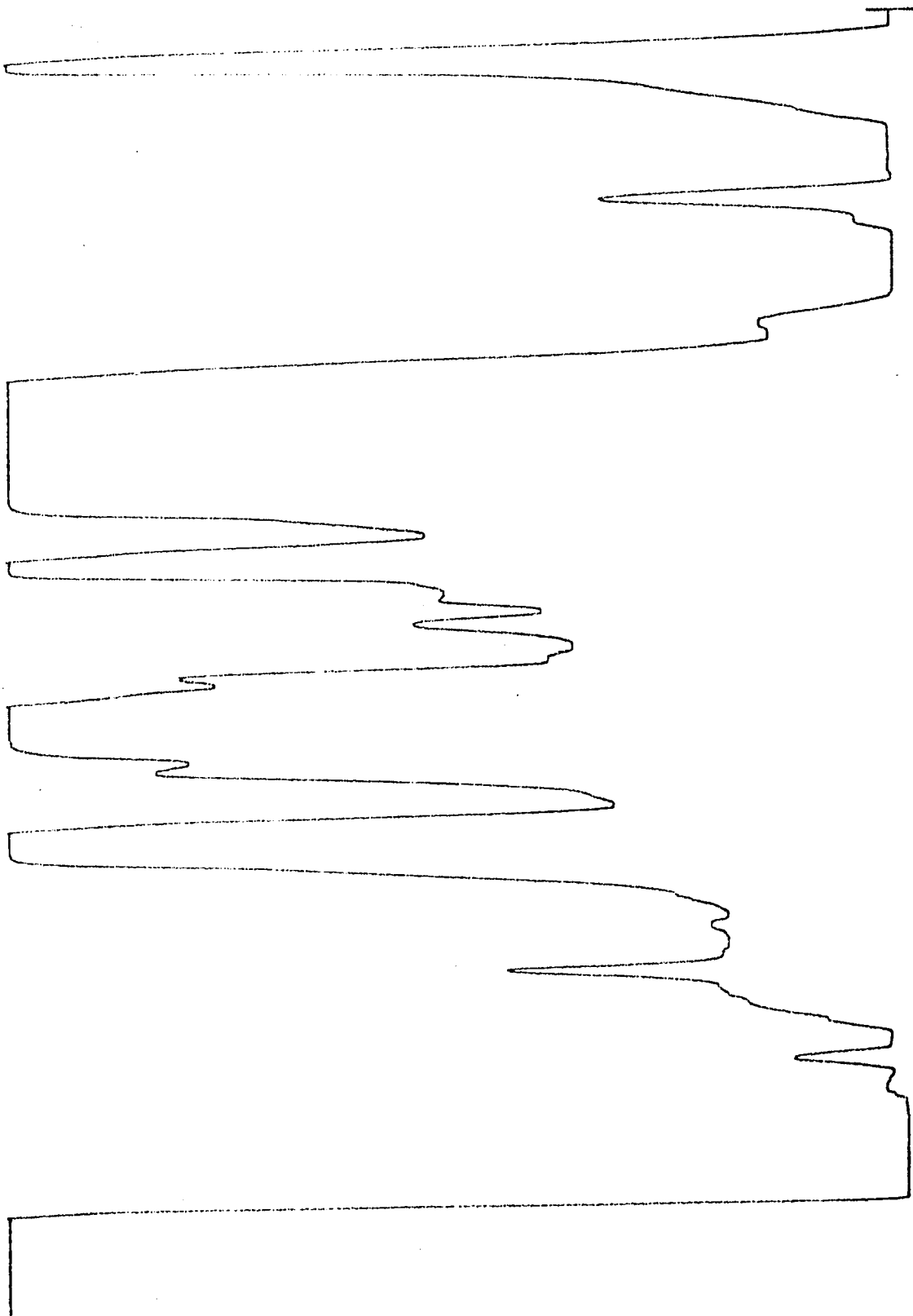
The difference between the banding patterns of normal and regressor sera versus the progressor sera was attributed to a very intense, fast moving band, which migrated about the same distance from the origin as the tracking dye. Employing the equation of Weber and Osborn (1969), a rough estimate of the molecular weight was calculated to be 37,000 daltons (Figure 13).

Because TSTA is present in both the lymphatic and venous circulation of individuals with progressing tumors (Smith, 1974) which might represent the blocking factor (Sjogren et al., 1971), disc-gel electrophoresis was performed with extracted TSTA (Halliday, 1971). Following electrophoresis, the banding patterns were compared with those obtained from the serum fractionization. Thirteen protein bands were evident; however, the fast-moving peak that appeared in the progressor sera was not evident in the crude TSTA following electrophoresis.

Lymphocyte washings from progressor (110 ug of protein) and normal (108 ug of protein) hosts along with

Figure 10. SDS-disc gel electrophoresis of normal chicken sera. A total of 640 ug of serum was applied to gels containing 7% acrylamide and run at 3 ma per tube at 5 C.

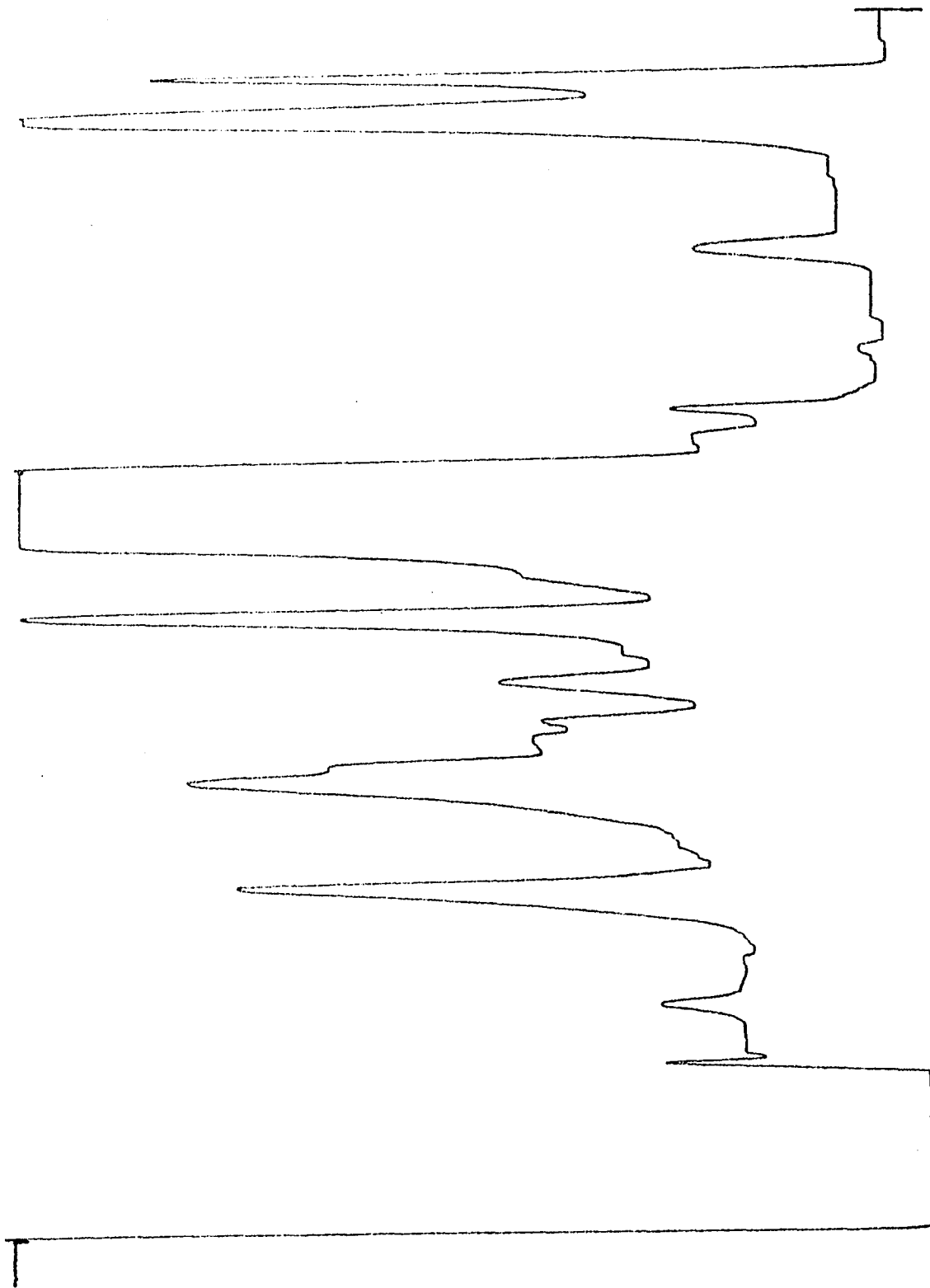
+



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Figure 11. SDS-disc gel electrophoresis of progressor chicken sera. A total of 400 ug of serum was applied to gels containing 7% acrylamide and run at 3 ma per tube at 5 C.

+



↓

Figure 12. SDS-disc gel electrophoresis of regressor chicken sera. A total of 380 ug of serum was applied to gels containing 7% acrylamide and run at 3 ma per tube at 5 C.

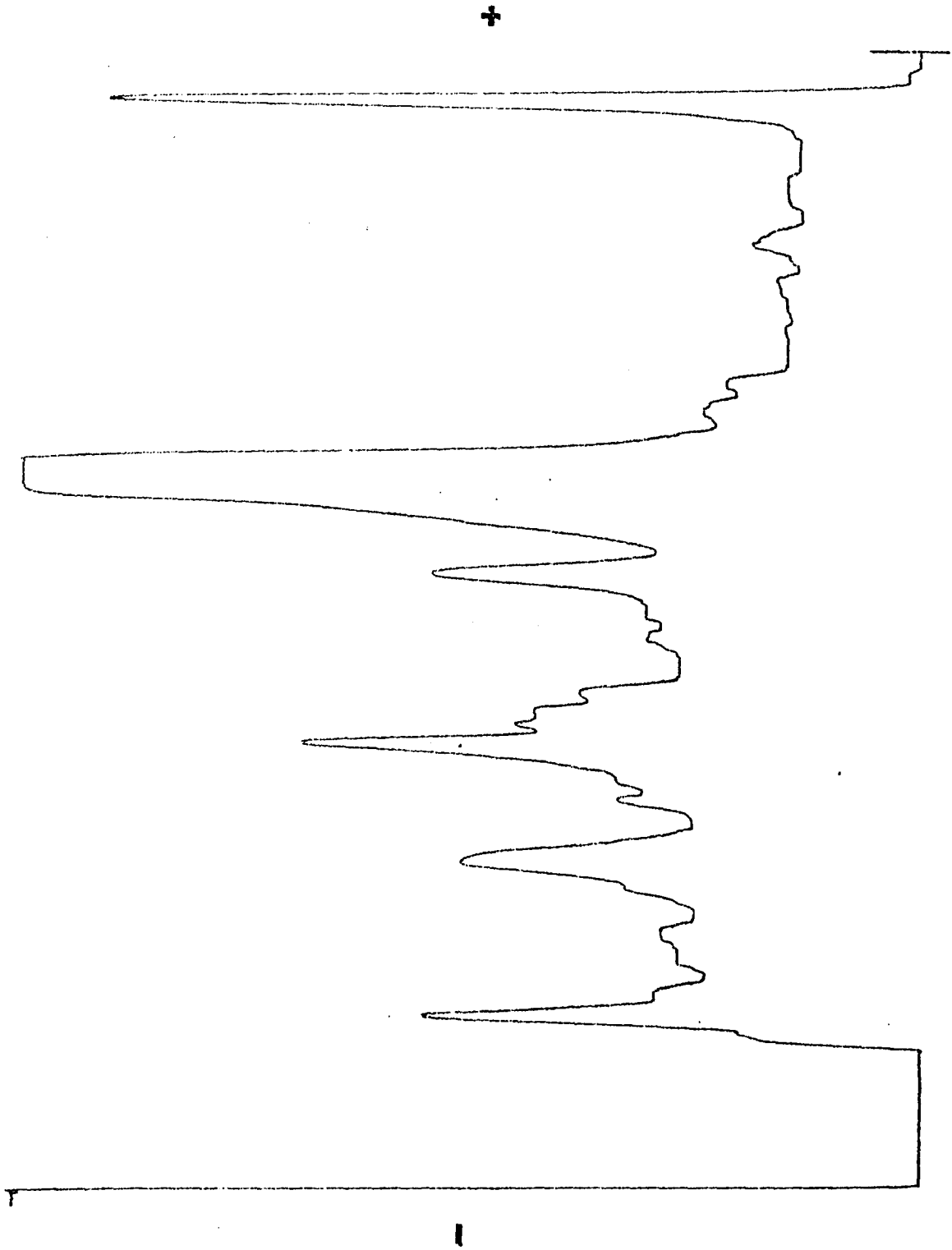
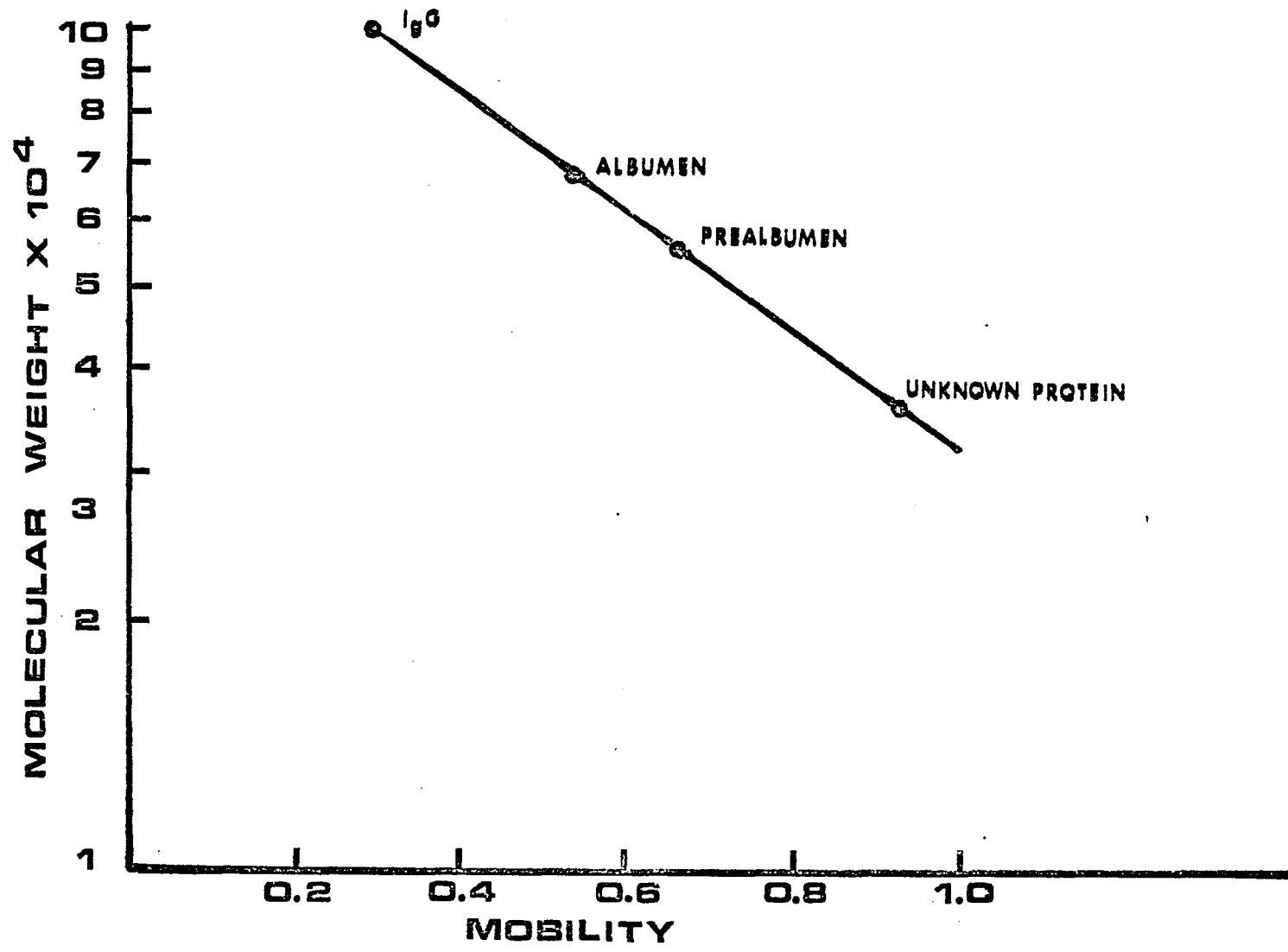


Figure 13. Molecular weight determination
of the unknown serum protein in
the progressor host.



TSTA were analyzed. Preliminary experiments indicated that eleven and thirteen faint bands were present in normal and progressor lymphocyte washings respectively. Thirteen bands were observed in the TSTA sample. The two additional protein bands associated with progressor lymphocyte washings were located approximately one-third the distance from the origin. Thus, these proteins are distinct from the highly mobile protein found in the serum.

DISCUSSION

ACRIDINE ORANGE STAINING

Staining of lymphocytes with acridine orange might prove to be an extremely valuable technique for diagnosing the presence or absence of cancer. In the studies described above, the fluorescence characteristics of lymphocytes obtained from chicken afflicted with RSV-induced tumors could readily be differentiated from those harvested from animals showing no signs of tumor or animals infected with other nononcogenic RNA viruses. In complete regressors, approximately 4% of the total lymphocytes fluoresced red before and after RNase treatment, suggesting that the RNA might be located intracellularly.

In all experiments conducted thus far, the percentage of red fluorescing lymphocytes from uninoculated hosts has been less than 0.5%. A peripheral (red fluorescing) lymphocyte count of 1% or greater would appear to indicate that the initial events in RSV oncogenesis had occurred since such levels were associated with tumor development. A decline of red fluorescing cells was associated with a subsequent decrease in tumor size.

With the virus inoculum used, virtually all inoculated hosts were prone to develop tumor. In two cases, however, inoculation of hosts with RSV did not lead to subsequent tumor formation and the percentage of red-fluorescing peripheral cells observed were similar to that obtained

with control chickens. Thus, these data imply that a direct relationship might exist between the red fluorescence phenomenon and tumor development.

It is of real interest that the initial response in the number of red fluorescing cells is parallel in all inoculated hosts for approximately the first 7 days. It is unlikely that this reflects a specific host defense response because those hosts having the greater percentages of red fluorescing cells developed relatively larger tumors. Rather the red fluorescing lymphocyte may result from deposition (on the lymphocyte surface) of a product formed by the virus-host interaction. Once the oncogenic process has been established and tumor increases in size, a product (RNA) might enter the circulatory system in increasing amounts and accumulate on the surface of a specific receptor lymphocyte. Sufficient RNA may accumulate at the periphery of this cell such that red fluorescence becomes evident. Ultimately, all the specific receptor cells become totally bound representing the observed 23% maximal fluorescence. Upon tumor regression, less material would be available for binding and corresponding decreases in fluorescing cells would be evident. Alternately, the observed response might indicate lymphocyte infection or possibly an antibody reaction with circulating virus at or near the surface of the lymphocyte.

That the cytotoxic activity of progressor lymphocytes is enhanced by washing suggests the removal of a sub-

stance from the cell surface (Currie and Basham, 1972). Our finding that the number of red fluorescing cells from progressors decreased significantly on washing could imply that the RNA associated with the surface of these lymphocytes might represent the substance inhibiting cellular immunity. Because RNase reduces the red fluorescing cell population of progressor chickens to levels found in normal uninoculated animals (i.e. 0.3%), nuclease treatment should (if our implication is correct) yield lymphocytes which are highly active against tumor cells. Such studies are to be undertaken and should reveal whether or not lymphocyte-associated RNA plays an important role in cellular immunity or simply represents a secondary effect of infection.

The RNase and washing studies provide evidence that the RNA associated with the progressor lymphocyte is located extracellularly, namely, at the cell surface. This observation is further substantiated by the finding that the nuclease is incapable of destroying the red fluorescence of lymphocytes obtained from complete regressors. What then is the origin of the RNA that is coating the cell surface? One remote possibility is that the polynucleotide is the viral genome itself. Hehlmann, Goldfeder and Spregelman (1974) demonstrated that during tumor progression, large amounts of mouse mammary tumor virus were released from transformed cells. A similar situation might be occurring in our system and the released virus might associate with the surface of sensitized lymphocytes and produce a red flu-

orescence when treated with acridine orange.

A difficulty arises with this hypothesis if one assumes that the viral genome is coated. Under these conditions, one would expect that the nuclease would be impervious to the intact particle. One might speculate, however, that the production of virus, or the association of virus with lymphocyte, in a progressor would result in an altered molecular configuration of the virion and permit entry of the nuclease into the virus core. One would also have to speculate however that such alterations do not occur in the regressing individual since RNase addition does not result in a loss of red fluorescence.

A more likely explanation for the origin of the RNA may be that 1) the polynucleotide originated from the tumor cell and may represent a product of the virus-cell interaction or 2) the RNA might represent a response product produced by another cell type, possibly the macrophage.

TUMOR-ASSOCIATED SERUM PROTEIN

Polyacrylamide disc-gel electrophoresis of progressor chicken sera has elucidated a protein band not present in regressor or normal hosts. This protein may represent a product of the neoplasia or a product of the virus-host interaction. My finding that the banding patterns obtained from the crude TSTA preparation differed from those of progressor sera indicates that circulating TSTA cannot account for the "neo-protein". One cannot rule out, however,

the possibility that a greater concentration of TSTA might exist in the circulatory system than in the actual tumor.

Likewise, it is unlikely that the "neo-protein" represents an immune response product (immunoglobulin). The immunoglobulin fraction in sera (IgG and IgM) is of larger molecule weight and bands out near the origin of the gel.

What then is the origin of this unique protein? This protein might represent a viral protein or a product of viral multiplication. As the tumor progresses to its maximum, the viral protein is at its maximum concentration. On the other hand, as the tumor regresses, viral multiplication is diminished or ceases and a concomitant loss of protein might occur from the circulatory system. It is apparent, however, that this "neo-protein" is associated only with individuals that possess maximal tumor.

RED FLUORESCENCE AND CYTOTOXIC ACTIVITY

The literature to date suggests that the agent involved in lymphocyte inactivation is either an antigen-antibody complex (Sjogren et al., 1971) or TSTA (Currie and Basham, 1972). Another possibility for lymphocyte "desensitization" is the association of an RNA-protein complex to the cell surface which results in loss of cytotoxicity. In the above studies treatment of washings with SDS followed by chloroform isoamyl alcohol, produce an increase in A_{260} material. This observation suggests that the RNA might be complexed with protein. Similar complexes, consisting of

antigen and messenger RNA have been isolated from a transformed hamster cell line (Mirault, Reed, and Stark, 1974) and a human cell line (Morel and Gardner, 1973; Lindberg and Sundquist, 1974). In the former case, the RNA-associated protein was not a segment of the polyribosomes as believed earlier (Perry and Kelley, 1968) but represented a "neo-protein" following transformation. Thus, an RNA-protein complex might be implicated in lymphocyte inactivation in hosts with progressive tumors.

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