and redissolved in 1 M NaCl. Removal of the uridine nucleotide by mild acid hydrolysis destroyed the serological activity and the uridine nucleotide removed was capable of inhibiting the C-CRP reaction, as was uridine monophosphate in a concentration of 1 mg/ml. It is suggested that the uridine nucleotide portion of the C substance is responsible for its complexing with human CRP.

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Production of Specific Neutralizing Antibody with Soluble Antigens of Type 5 Adenovirus.* (28579)

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Biosynthesis of 3 well-defined soluble antigens and infectious virus follows adenovirus infection of a number of mammalian cells *in vitro*(1,2). The soluble antigens, which are readily separable from mature virus particles as well as from each other(1,2,3), are predominantly protein in nature(4) and have been shown to represent virus structural subunits produced in excess(4,5,6,7). To obtain additional evidence on the relationship of the soluble antigens to the virus proteins, advantage was taken of the fact that injection of the purified soluble antigens into rabbits leads to the production of specific antibodies directed against the type-specific (E) or group-specific (L) soluble antigens present in type 5 virus-infected cells(2). The investigation described here had as its objective to characterize further the nature and role of the E and L soluble antigens by immunological means, employing antigen-specific antisera in neutralization, complement-fixation, and cross-adsorption tests for this purpose. The data thus obtained strengthen the evidence that the soluble antigens represent virus subunits which have been made in excess but have not been incorporated into virus particles.

Materials and methods. Tissue culture. HeLa cells and, on occasion, KB cells(8) propagated in continuous serial culture as previously described(9) were employed. For infectivity and neutralization titrations cul-

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tures were prepared in screw-capped 13 x 150 mm test tubes and maintained in a minimal medium(9).

Preparation of crude virus and antigen pools. Mixtures of virus and soluble antigens were prepared as previously described(2) by infecting large monolayer cultures with the desired adenovirus type. Infected cells were incubated at 36°C until cytopathic effects were complete (5 to 6 days) at which time the cells were concentrated from the maintenance mixture by centrifugation at 1000 rpm for 20 minutes and washed 2 times with 0.15 M NaCl buffered with 0.01 M phosphate at pH 7.2 (PBS) warmed to 37°C. Washed cells, resuspended in PBS at one-tenth the original volume were disrupted by homogenizing at 16,000 rpm in a Servall Omnimixer. Cell debris was removed by a single cycle of centrifugation in a Model L Spinco preparative centrifuge at 6,600 $\times$ g for 15 minutes. The supernatant fluid containing both virus and soluble antigen, referred to hereafter as crude virus, was stored at a –28°C until used.

Preparation of antisera. Rabbits were given 3 intramuscular injections of antigens in Freund's adjuvant at weekly intervals followed a week later by one intraperitoneal injection of antigen without adjuvant. Animals were bled 12 to 14 days after the final injection. Sera thus obtained were inactivated at 56°C for 30 minutes and stored at –28°C.

Neutralization titrations. To serial 2-fold dilutions of serum in Hanks' balanced salt solution (BSS) was added an equal volume of BSS containing 100 ID_{50} of the appropriate adenovirus type. Serum-virus mixtures were incubated for 30 minutes at 37°C. For each serum dilution, 0.2 ml of serum-virus mixture was inoculated into each of 3 culture tubes. Inoculated cultures were incubated at 36°C for 7 days. The end point was considered to be the highest final dilution of serum in the serum-virus mixture which prevented cytopathic changes in one-half or more of the HeLa cells in each group. The Reed-Muench calculation at the 50% end point was employed.

Infectivity titrations. Titrations were carried out in tube cultures of HeLa cells using $10^{-0.5}$ (1:3.2) dilution increments as previously described(2).

TABLE I. Preparation of Virus-Free Soluble Antigens.

Description of sample	Infectivity titer (log_{10})*	Complement-fixing titer†
Original crude virus	$10^{-8.2}$	8192
Supernate, 1st centrif.‡	$10^{-5.0}$	8192
" , 2nd "	$10^{-2.2}$	8192
" , 3rd "	<10^{0}§	8192
" , 4th "	<10^{0}§	8192

* Titer per 0.2 ml inoculum.

† Reciprocal of the highest antigen dilution yielding complete fixation of complement.

‡ Centrifugation was carried out at 105,000 $\times$ g for 90 min.

§ No infectious virus was found in undiluted material.

Complement-fixation titrations. A standard test was employed utilizing 1.5 complete units of complement. Heat-inactivated (56°C for 30 minutes) virus type-specific or antigen-specific rabbit antisera were used throughout. Titers are expressed as the greatest dilution of antigen or serum, giving complete fixation.

Experimental results: Preparation of virus-free antigens. Crude virus pools, prepared as described, were subjected to 4 consecutive cycles of ultracentrifugation, each at 105,000 $\times$ g for 90 minutes. At the end of each cycle of centrifugation the top one-half of the supernatant fluid was carefully removed from each tube, pooled, and a representative sample saved for infectivity and complement-fixation titrations. The results of these titrations, presented in Table I, indicate that no detectable virus remained in the antigen mixture after this procedure. Following ultracentrifugation, the antigen mixture was chromatographed on DEAE-cellulose columns. By employing a step-wise (non-continuous) pattern of elution with buffered NaCl solutions of increasing molarity, it was possible to separate and obtain individually each of the 3 soluble antigens characteristically produced by type 5 virus-infected cells(2). To insure the homogeneity of these antigens, each was re-chromatographed 3 times under appropriate conditions of pH and NaCl concentration. It is important to note that a single passage of a soluble antigen preparation through a column of DEAE-cellulose at a NaCl concentration less than 0.3 M removed greater than 99% of any infectious virus that might be

TABLE II. Reactivity of Antigen-Specific Antisera with Type 4 and Type 5 Adenovirus Antigens.

Antigen employed	Complement-fixation titer with antiserum*	
	Anti-E_5	Anti-L_5
Type 5, E†	1:128	<1:16
" 5, L†	<1:16 §	1:128
" 5, T†	1:32	<1:16 §
" 4, E + L‡	<1:16 §	1:64

* Expressed as the reciprocal of the greatest dilution of serum that effected complete fixation of 1.5 units of complement in the presence of a standard amount of the indicated antigen.

† E = type-specific antigen; L = group antigen; T = toxin. The T antigen was contaminated with type-specific antigen (E).

‡ Consists of a virus-free mixture of specific (E) and group (L) antigens from type 4 adenovirus-infected cells.

§ Serum dilutions less than 1:16 were anticomplementary.

present. It can be deduced, therefore, that 4 cycles of chromatography removed whatever small undetectable amounts of virus that might have persisted in the antigen preparations after ultracentrifugation. Following the final cycle of chromatography, the antigen solutions were dialyzed against 0.01 M phosphate buffer, pH 7.2, and lyophilized. Dried material was reconstituted to the desired volume in sterile PBS and employed to immunize rabbits. During the course of immunization, individual animals received either 8 mg of type-specific (E) antigen protein, 2 mg of toxin protein, or 5 mg of group-specific (L) antigen protein. Lyophilized antigen preparations were reconstituted without detectable loss in complement-fixing or toxin potency.

Specificity of antisera produced in response to virus-free antigens as determined by complement-fixation. Antisera prepared in rabbits following injection of soluble virus-free antigens were tested for their ability to fix complement with each of the purified antigens. The results of these experiments, shown in Table II, indicate that the E and L antigens of type 5 virus exhibited no cross-reactions by the immunological procedures employed. The group specificity of the L antigen is indicated by the reaction of the anti-L with antigens derived from type 4 adenovirus-infected HeLa cells. The toxin (T antigen) employed was contaminated with a relatively large amount of E antigen. This observation is not surprising since the elution optima of these antigens from DEAE-cellulose is quite close. Therefore, data obtained with antisera prepared by immunization of rabbits with toxin were ambiguous and are not presented. However, it should be noted that anti-E serum did not neutralize toxin whereas anti-T effectively neutralized the cytopathic effects of toxin at dilutions as high as 1:256 and greater.

Capacity of antigen-specific antisera to neutralize virus. Antigen-specific antisera prepared from virus-free type 5 antigens were tested for their ability to neutralize type 5, type 4, and type 2 adenoviruses. The procedures used are described in the section on methods. For purposes of comparison and control, neutralization titrations were done with antisera obtained from rabbits immunized with type 5 virus and from rabbits immunized with the same virus suspension that prior to injection had been diluted to a final virus concentration of approximately 10 ID_{50} per ml. From the results of these experiments, summarized in Table III, it is apparent that the adenovirus-induced, soluble antigens elicited the production of type-specific virus neutralizing antibody. The observation that antisera prepared in response to the injection of a dilute, crude virus suspension failed to neutralize virus makes it extremely unlikely that the antigenic potency of the soluble antigens could be related to the presence of very small amounts of virus not detectable by the assay procedure employed. It is striking that injection of the

TABLE III. Production of Specific Neutralizing Antibody with Virus-Free Antigens from Type 5 Adenovirus-Infected Cells.

Antiserum prepared with	Neutralizing titer with antigen*		
	Type 5 virus	Type 4 virus	Type 2 virus
Type 5, E antigen	1:512	<1:16	<1:16
" 5, L "	1:2048	<1:16	<1:16
Crude type 5 virus	1:1024	<1:16	<1:16
Diluted type 5 virus†	<1:16	<1:16	<1:16

* The greatest dilution of serum which prevented cytopathic changes by 100 ID_{50} of virus in one-half or more of the HeLa cells in each group.

† Crude type 5 virus suspension diluted to a final virus concentration of 10 ID_{50} /ml.

group-specific L antigen elicited the production of antibody which was highly type-specific as measured by neutralization titrations, but group-specific when tested by the complement-fixation technique. One cannot, however, on the basis of these data, discount the possibility that more than one antibody was involved in these reactions. Antisera prepared in response to the group-specific or L antigen consistently exhibited greater neutralizing capacity than did antisera directed against the type-specific E antigen.

Further properties of the L antigen as revealed by cross-adsorption studies. The finding that injection of the group-specific or so-called "common" adenovirus antigen elicited the formation of a type-specific neutralizing antibody was unexpected. It was considered that if the group-specific or L antigen was actually a "common" antigen produced by all adenovirus-infected cells, the neutralizing antibody elicited in response to the injection of this antigen should also be group-specific. With this in mind, efforts were made to characterize further the antigenic properties of the L or so-called group-specific antigen.

Antiserum made in rabbits by injection of purified L antigen obtained from type 5 virus-infected HeLa cells (Anti-L_5) was adsorbed with either purified homologous (type 5) or partially purified heterologous (type 4 or type 2) L antigen. Equal amounts of these antigens, about 50,000 complement-fixing units, were employed for adsorption. Antiserum at a dilution of 1:5 was added directly to lyophilized antigen to avoid dilution effects. Desiccated HeLa cell extract was used as a control. After 2 cycles of adsorption overnight at 4°C, the serum-antigen mixtures were heated at 61°C for 15 minutes and centrifuged to remove the insoluble precipitate. This procedure greatly decreased the anti-complementary properties of the adsorbed sera. Such sera were tested in a progressive series of 2-fold dilutions for their ability to fix complement with a constant amount of type 5, type 4, or type 2 derived L antigen. The appropriate antigen concentrations for each adsorbed serum were predetermined by means of a "box-titration." The results of these experiments, shown in Table IV, are expressed as the reciprocal of the greatest dilution of adsorbed serum leading to complete fixation of complement with the designated antigen. It is apparent from an inspection of these data that: 1) adsorption of anti-L_5 with normal cell protein did not impair its capacity to react with either homologous or heterologous L antigens; 2) adsorption of anti-L_5 with type 5-derived L antigen completely abolished the capacity of the adsorbed serum to react with either homologous or heterologous antigen; 3) adsorption of anti-L_5 with either type 4 or type 2 L antigen resulted in total adsorption of that component of the serum which reacted with L antigen derived from heterologous sources but had little effect upon the ability of the serum to fix complement with the homologous or type 5-derived L antigen.

TABLE IV. Effect upon the Complement-Fixation Titer of Anti-L_5 Serum by Adsorption of Serum with Group Antigen Derived from Type 5, Type 4, or Type 2 Infected Cells.

Serum	Antigen used for adsorption	Complement-fixation titer† of adsorbed sera with antigen		
		Type 5 L	Type 4 L	Type 2 L
Anti-L_5	None	160	80	160
" -L_5*	Type 5 L	<5	<5	<5
" -L_5	" 4 L	160	<5	5
" -L_5	" 2 L	80	<5	<5
" -L_5	NCE‡	160	80	160

* Antiserum directed against type 5-induced L antigen.

† Expressed as the reciprocal of the greatest serum dilution causing complete fixation of 1.5 units of complement with a constant amount of antigen.

‡ Normal cell protein.

Efforts to correlate these data with an effect upon the virus neutralizing capacity of the anti-L_5 serum have been difficult. Adsorption of serum by highly purified type 5 L antigen greatly decreased the neutralizing capacity of the anti-L_5 serum. However, the presence of substantial amounts of the E or type-specific antigen (difficult to separate from group antigen on DEAE(2)) in the type 4 and to a lesser extent the type 2 preparations interfered markedly with determinations in cultures containing the serum-E antigen mixtures. The suppressive effect of the E or type-specific antigen upon virus multiplication has been previously noted by Pereira

(10) and confirmed in this laboratory.

Discussion. Infection of cells by adenoviruses induces the biosynthesis of 3 distinct antigens separable from the virus particles: the type-specific (E) antigen which characterizes the immunological specificity of each type; the common, group (L) antigen which defines the agent as an adenovirus; and the toxin-like material (T) which likewise reacts as a common group antigen(1,2). Evidence obtained from investigation of the kinetics of biosynthesis of these antigens, utilizing direct chemical measurements as well as inhibitors of protein synthesis, suggested that the soluble antigens were precursors of the virus protein and probably the antigenic subunits of the virus particle(6,7,11). The hypothesis that the soluble antigens are precursors of virus protein has now been demonstrated by immunologic and electron microscopic techniques(4,5). Moreover, the group L antigen was shown to be identical to the capsomeres of the virus particle, and the type-specific E antigen was demonstrated to be the same as a fiber-like structure obtained by disruption of the virus particle(5). The L antigen is the major antigenic component of the virus particle being present in a concentration 15-20 times greater than the type-specific antigen (4). These data clearly indicate that the soluble antigens, synthesized before the infectious virus particles, are virus subunits which accumulate in the infected cell because they are produced in excess of those actually assembled into virus particles.

The evidence that the L antigen is the predominant antigen of the virus particle and in addition represents the major structural component, the capsomeres, of the virus clarifies and makes less surprising the finding that L-specific antibody neutralizes virus infectivity. However, these experimental facts did not offer an explanation for the finding that the purported "common group" antigen obtained from type 5 adenovirus neutralized only the homologous type virus. The concept that the L antigen is a common group antigen is based upon evidence obtained with: 1) complement fixation titrations utilizing crude virus preparations, and convalescent human serum or serum from rabbits immunized with crude virus; and 2) complement fixation or gel-diffusion techniques employing purified antigens and serum from rabbits immunized with infected cell homogenates. That the so-called common group antigens were not identical in all types of adenoviruses was clear from the finding that the elution characteristics from DEAE-cellulose differed with various types(2,12). Cross- adsorption studies utilizing purified antigen-specific antisera and purified antigens freed from intact virus particles lend additional strong support to the postulate that the L antigen is immunologically different for each virus type: the L antigen cross-reacts extensively with a similar antigen associated with each type virus, but in addition has type-specificity.

Two alternative interpretations can be presented to explain these data: 1) The so-called common group antigen, the L antigen, may have 2 antigenic determinants on the same molecule, one which contributes group reactivity and one which is unique to the L antigen of each type and, therefore, it endows the L antigen with a degree of type-specificity which is most clearly manifest by the neutralization reaction. In this context, it is striking that the "type-specific reactive site" of the L antigen does not cross-react with antiserum to the type-specific E antigen, nor does adsorption of anti-E serum with purified L antigen of the homologous type diminish the capacity of the adsorbed serum to react with the type-specific E antigen. This hypothesis implies that the type 5 agent possesses 2 structurally different type-specific determinants. Therefore, neutralizing antibodies made in response to type 5 virus infection would consist of 2 species of antibody, each possessing a different specificity and each reacting independently of each other. 2) The L antigen stimulates production of a heterogeneous population of antibodies of which less than 50% can cross-react with heterologous antigens. Because of structural differences between the homologous and heterologous antigens, the majority of antibodies react with greatest affinity for the homologous antigen and the cross-reacting antibody-antigen reaction has a considerably lower affinity

constant. Inherent in this notion is the assumption that neutralization of virus requires high affinity type antibody. These characteristics would permit the observed cross-reaction as measured by complement-fixation, the adsorption of only cross-reacting antibodies by heterologous antigens, and the type-specific reaction as determined by neutralization titrations. Data are not yet available to select between these two hypotheses, and each has attractive features which can be experimentally investigated. Regardless of which of these postulates is correct, it must be emphasized, however, that the purported "group-antigen," the L antigen is distinct for each type although it does cross-react with heterologous anti-L antibodies.

The experimental findings described present further evidence on the immunological structure of adenoviruses. These data suggest that immunity following an adenovirus infection is stimulated not only by the virus particles which are synthesized in the host but also by the soluble antigens which accumulate in infected cells. That soluble antigens can initiate synthesis of neutralizing antibodies is not a phenomenon restricted to adenoviruses: Appleyard recently demonstrated that a soluble antigen from rabbit-pox or vaccinia virus could elicit the formation of neutralizing antibodies in rabbits and mice(13). It may be deduced from these data that artificial immunization can be produced not only by virus particles but also, and perhaps more effectively, by utilization of purified soluble antigens.

Summary. Injection into rabbits of virus-free soluble antigen preparations obtained from type 5 adenovirus-infected HeLa cells results in production of specific virus neutralizing antibody. The neutralizing antibodies are present in antisera in high titer and are completely type-specific. This is interpreted as evidence that the virus-induced soluble antigens are structurally related to virus protein. Adsorption studies employing purified group-specific (L) antigens derived from types 2, 4, and 5 virus-infected cells and antiserum specific for the type 5 L antigen reveal basic immunological differences in these "common group" antigens: The type 5 L antigen cross-reacted with type 2 and 4 antigens; however, the type 5 antigen, in addition, had an antigenic reactivity which was type-specific.

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