er similar prokaryotes, such as the genus *Prochlorion*, which contains chlorophyll b (*8*). This interpretation is confirmed by studies of nucleotide sequences in 16*S* RNA's (*18*). A large chloroplast genome of the order of 1000×10^6 daltons that approaches the bacterial genome in size is what one might expect to find in the truly primitive chloroplast according to the endosymbiont theory. Indeed, the large molecular size of 1500×10^6 daltons reported for ctDNA in *Acetabularia* has been interpreted as evidence for the primitive nature of this genome (*3*). Our discovery of an exceptionally small chloroplast genome (56×10^6 daltons) in *Codium* raises the alternative possibility that small genome size may be primitive in extant species of green plants, and underscores the need for an extended survey of the properties of ctDNA in algae.

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Viral Epitopes and Monoclonal Antibodies: Isolation of Blocking Antibodies That Inhibit Virus Neutralization

Abstract. *The inability of pathogenic animal viruses to be completely neutralized by antibodies can lead to chronic viral infections in which infectious virus persists even in the presence of excess neutralizing antibody. A mechanism that results in this nonneutralized fraction of virus was defined by the topographical relationships of viral epitopes identified with monoclonal antibodies wherein monoclonal antibodies bind to virus and sterically block the binding of neutralizing antibodies.*

Interactions of a wide variety of viruses with neutralizing antibodies usually result in a small fraction of the viral population escaping neutralization even in the presence of excess antibody (*1*). The biological importance of this phenomenon derives both from observations that infectious viruses can persist in animals in the presence of neutralizing antibody (*2*) and from its implications for persistent viral infections in a wide array of systems including those of significance to man (*3*). Although the mechanism for the generation of the nonneutralized fraction is not completely understood, one of several mechanisms suggested by Dulbecco *et al.* (*1*) was that antiserum contained uncharacterized molecules, presumably antibodies, which combined with viruses so as to inhibit the attachment of neutralizing antibody without inhibiting virus infectivity. It was subsequently shown (*4*) that the nonneutralized fraction of virus could be neutralized by the addition of a secondary antibody specific for the primary antibody. This result implied that the surviving virus had reacted with primary antibody but had remained infectious. To understand how one antibody can bind virus and not neutralize it but can interfere with the binding of a second antibody that does neutralize, requires knowledge both of the topography of the combining sites for each antibody and the mechanism of antibody-mediated virus neutralization.

Because of their homogeneity and spe-

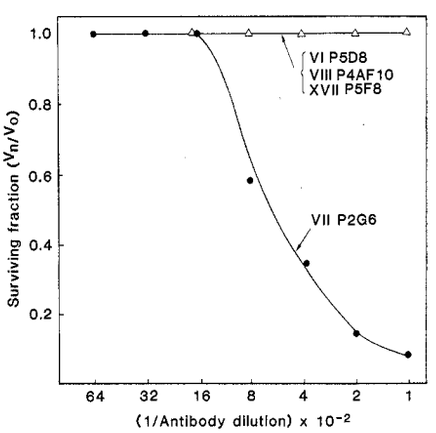

Fig. 1. Neutralization of KiSV (C3H MMTV) pseudotype with monoclonal antibodies. Because there is no direct quantitative infectivity assay for MMTV in vitro, neutralization was measured by the ability of the various monoclones to neutralize the focus-forming capacity of Kirsten sarcoma virus (KiSV) C3H MMTV pseudotype. This pseudotype contained the envelope glycoprotein gp52 of C3H MMTV and it has been previously shown that gp52 is a target for neutralization (*6*). Hybridomas producing monoclonal antibodies to C3H MMTV were isolated as described (*7*) from cell clones derived from a fusion between lymphocytes from an immunized mouse and the NS-1 myeloma cell line (*8*). All the monoclones were judged to be reactive with gp52 on the basis of radioimmunoprecipitation assays (data not shown). The antigenic specificities of these antibodies are: VII P2G6 (IgG_{2a}) type-specific, reactive only with the C3H strain of MMTV; VI P5D8 (IgM) and VIII P4AF10 (IgG_3), class-specific, reactive with C3H and GR strains of MMTV's; XVII P5F8 (IgG_3), group-specific, reactive with C3H, GR, RIII, and C3Hf strains of MMTV's. Ascites fluids containing each of the monoclones at a 1:100 dilution were heat-inactivated at 56°C for 30 minutes and filtered. Serial twofold dilutions were mixed with 200 focus-forming units of virus and incubated at 37°C for 30 minutes. Virus-antiserum mixtures were added to duplicate 60-mm dishes containing Fisher rat embryo cells as described (*5*). Foci appeared between 10 and 14 days. The number of foci induced in the presence of antibodies (V_n) and the number of foci induced in the absence of antibodies (V_o) were used to calculate the surviving fraction.

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cific reactivity with individual antigenic determinants (epitopes), monoclonal antibodies (monoclones) are ideal probes for studying the spatial relationships or topography of viral epitopes and their function as targets for neutralization. Using monoclones to the major external glycoprotein (gp52) of mouse mammary tumor virus (MMTV, a retrovirus that induces mammary adenocarcinomas in mice), we recently defined the topographical sites and mechanisms for antibody-mediated virus neutralization (*5*). On the basis of the results of that study, we began to look for monoclones that could react with virus and produce a nonneutralized fraction.

In the previous study (*5*), we defined two topographically distinct sites on MMTV gp52. The first topographical site functioned as the target site for neutralizing antibody and was defined by the observation that all monoclones that neutralized virus infectivity also competed for binding of a neutralizing monoclone (VII P2G6). The second site bound monoclones but was not a target for neutralization. This site was topographically distinct because these monoclones did not compete for the binding of VII P2G6.

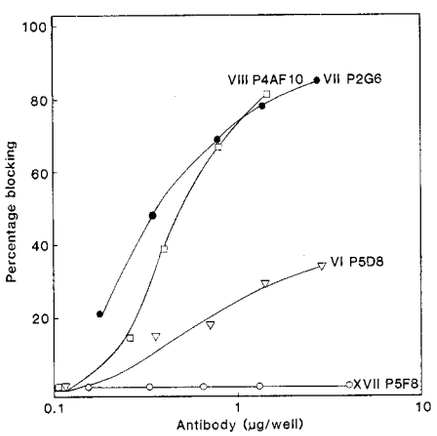

Fig. 2. Blocking of ^{125}I-labeled monoclone VII P2G6 binding to C3H MMTV with unlabeled monoclones. C3H MMTV was adsorbed to individual wells of microtiter plates at a concentration of 2 μg per well. Further nonspecific binding of proteins to the wells was eliminated by adding 150 μl of bovine serum albumin (5 percent BSA in phosphate-buffered saline, *p*H 7.2) as described (*7*). Fifty microliters of serial twofold dilutions of ascites fluids (starting at a 1:100 dilution) were incubated in each well for 45 minutes at 37°C and then 4×10^5 count/min of ^{125}I-labeled purified VII P2G6 was added and incubated for an additional 45 minutes at 37°C. Monoclone VII P2G6 was purified from ascites fluids on a protein-A sepharose column and iodinated by the chloramine T method (*5*). The amount of ^{125}I-labeled VII P2G6 bound to each well was determined by eluting with 2*N* NaOH and counting the eluate in a gamma counter. The amount of unlabeled competitor antibody added to each well was extrapolated from the concentration in the ascites fluids determined by radial immunodiffusion.

It was further shown that neutralizing antibody prevented virus adsorption to cells by binding to epitopes adjacent to the determinants that function in the binding of virus to cell surface receptors. Therefore, the VII P2G6 domain (defined by those antibodies that compete for VII P2G6 binding) overlapped with the domain defined by the monoclones that neutralize and sterically hinder virus-cell receptor binding. It was postulated that the VII P2G6 epitope was probably not the receptor binding site but was adjacent to it. We therefore predicted the existence of epitopes within the VII P2G6 domain but not within the virus receptor binding domain. Monoclones to these postulated epitopes would compete for the binding of neutralizing antibody VII P2G6 but would themselves not neutralize virus infectivity. A consequence of binding of these blocking antibodies would be the protection of virus particles from neutralization.

In the present study, a wider panel of monoclones was tested for their ability to compete for ^{125}I-labeled VII P2G6 binding to MMTV and to neutralize virus infectivity. Antibodies that gave discordant results, that is, did not neutralize yet competed for VII P2G6 binding, would be potential blocking antibodies. Competition assays were performed in which we used ^{125}I-labeled VII P2G6 and a panel of unlabeled monoclones as competitors. Increasing concentrations of unlabeled monoclones were added to wells in which the C3H strain of MMTV (C3H MMTV) was bound. The ^{125}I-labeled VII P2G6 monoclone was then added and the amount bound was measured and used to determine which monoclones competed for binding of the labeled antibody. Neutralization was measured by the ability of the various monoclones to neutralize the focus-forming capacity of a Kirsten sarcoma virus (C3H MMTV) pseudotype, KiSV (C3H MMTV). This pseudotype contained MMTV gp52 in its envelope and this was demonstrated to be a target for neutralizing antibodies (*6*). Two out of a total of 13 monoclones tested competed for the binding of ^{125}I-labeled VII P2G6 and did not neutralize the KiSV (C3H MMTV) pseudotype. Figures 1 and 2 show the results for these two monoclones (VI P5D8 and VIII P4AF10). For comparison, the results for a nonneutralizing, noncompeting monoclone (XVII P5F8) and for a neutralizing and competing monoclone VII P2G6 are also presented. The inability of VI P5D8 and VIII P4AF10 monoclones to neutralize MMTV was not due to low binding avidities because their binding avidities were comparable to monoclone VII P2G6 (data not shown) and they could effectively compete for the binding of VII P2G6 (Fig. 2). The inability of XVII P5F8 to either compete or neutralize resulted from its binding to a topographical site distinct from the domains involved in cell-receptor and VII P2G6 binding, as previously shown (*5*).

These results demonstrate that the domains for monoclones VI P5D8 and VIII P4AF10, but not for XVII P5F8, overlap with the domain defined by VII P2G6 and do not overlap with the receptor binding domain. Therefore, as previously predicted, the VII P2G6 domain must lie adjacent to the receptor binding site,

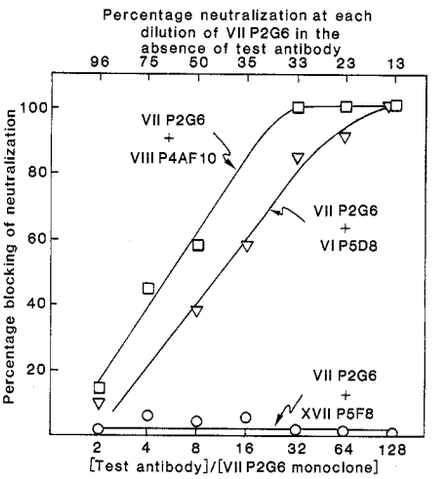

Fig. 3. Blocking of neutralization by VII P2G6 with monoclones that compete for its binding to MMTV. KiSV (C3H MMTV) was first incubated with 1:100 dilutions of test antibody VI P5D8, VIII P4AF10, XVII P5F8, or medium alone for 30 minutes at 37°C and then with serial twofold dilutions of VII P2G6 (1:200 to 1:12,800). The individual ascites fluids used for this experiment contained comparable concentrations of antibody as determined by radial immunodiffusion assays. Virus-antiserum mixtures were added to duplicate 60-mm dishes of Fisher rat embryo cells and incubated at 37°C for 7 to 10 days. The number of foci were quantitated and used to calculate the percentage neutralization and percentage blocking of neutralization of VII P2G6. The percentage neutralization at each dilution of VII P2G6 in the absence of other antibody was calculated from the equation: percentage neutralization = $[1 - (V_n/V_o)] \times 100$. The percentage blocking of neutralization at each ratio of test antibody to VII P2G6 was calculated from the equation: percentage blocking of neutralization = [1 − (percentage neutralization in the presence of test antibody divided by the percentage neutralization in the absence of test antibody)] × 100.

and VI P5D8 and VIII P4AF10 monoclones should be capable of blocking virus neutralization. To test this, KiSV (C3H MMTV) was first incubated with a 1:100 dilution of either VI P5D8 or VIII P4AF10 for 30 minutes at 37°C and then with serial twofold dilutions of VII P2G6 monoclone. Virus-antiserum mixtures were added to duplicate dishes containing indicator cells and incubated for 7 to 10 days at which time the number of foci was quantitated. As a control, the nonneutralizing, noncompeting monoclone XVII P5F8 was used in the first incubation period. The results are presented in Fig. 3. As expected, only monoclones VI P5D8 and VIII P4AF10 were able to block the neutralizing capacity of monoclone VII P2G6. In contrast, monoclone XVII P5F8 has no effect on the neutralizing capacity of VII P2G6. The results show that the percentage blocking increases as the concentration for each blocking monoclone increases relative to that for VII P2G6.

The use of monoclonal antibodies for topographical analysis represents an important new approach for identifying the mechanism of antibody-mediated virus neutralization and the generation of the nonneutralized fraction. Although the nonneutralized fraction has been observed with other viruses and polyvalent antiserums, to our knowledge this is the first direct demonstration that this occurs by way of blocking antibodies. This mechanism must now be considered in studying chronic viral infections in which infectious virus persists in the presence of neutralizing antibodies. Examples of chronic viral infections in humans that fit into this category are chronic viral hepatitis, subacute sclerosing panencephalitis (measles virus), and Epstein-Barr virus infections. The results of this study should be applicable for modifying such disease states. Finally, in designing future vaccination programs with purified antigens, fragments of antigens, and adjuvants, the potential for generating blocking antibodies should be strongly considered.

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Evaluation of Nitrate Synthesis by Intestinal Microorganisms in vivo

Abstract. *The nitrate balance of germfree and conventional rats was assessed to determine whether the intestinal flora produces nitrate in vivo. The results indicate that there can be excess nitrate in the urine of germfree as well as conventional rats. This nitrate is apparently of host origin, and the presence of intestinal flora decreases the output of nitrate in urine.*

It is now widely accepted that *N*-nitroso compounds, whether of exogenous or endogenous origin, may be involved in the etiology of several types of human cancer (*1*). The pharmacokinetics of nitrite and nitrate are important considerations, since these compounds, under appropriate conditions, can nitrosate amines and amides to form the corresponding *N*-nitroso compounds.

Tannenbaum and his colleagues have reported that humans (*2*) and rats (*3*) have more nitrate in their urine than they ingest in their diet and that nitrate and nitrite are present in the intestinal contents of humans (*2*). To explain the excess urinary nitrate and the presence of these ions in the intestinal contents, it was proposed (*2*) that heterotrophic microorganisms in the intestinal tract synthesize nitrate and nitrite from reduced nitrogenous compounds by a process termed heterotrophic intestinal nitrification (HIN). In support of this concept, it was reported (*4*) that microorganisms isolated from the human intestinal tract are able to oxidize certain nitrogenous compounds to nitrite.

Heterotrophic intestinal nitrification, if it occurs, is important for the following reasons.

1) Oxidation of organic or inorganic nitrogenous compounds to nitrite or nitrate by heterotrophic bacteria in the intestinal tract has not previously been described. These bacteria would be conducting a novel reaction at an incredible pace to make excess nitrate possible.

2) Intestinal synthesis of nitrite or nitrate could account for the *N*-nitroso compounds reported in feces (*5*), since intestinal bacteria can nitrosate amines in the presence of these ions (*6*). Such *N*-nitroso compounds could explain the etiology of colorectal cancer.

3) Concerns over the carcinogenicity of exogenous nitrite (*7*), such as that added to processed foods, would seem to be unfounded, since it has been suggested that thousands of times more nitrite is synthesized in the intestinal tract than could be ingested with food (*8*).

4) Intestinal synthesis of nitrite or nitrate could play a role in the etiology of other types of human cancer, not only by increasing the amount of *N*-nitroso compounds in the body but also through direct carcinogenic action (*7, 9*).

5) Various *N*-oxy and *N*-hydroxy compounds, which could be intermediates of microbial HIN, have antimicrobial activity (*10*) and might play an important role in controlling the microbial ecology of the intestinal tract.

Earlier (*11*), we offered alternative explanations for the presence of nitrite and nitrate in the intestinal tract, argued (*12*) that HIN is unlikely, and suggested other sources of urinary nitrate. This report provides further data relating to the nondietary origin of nitrate in rats.

If intestinal bacteria or fungi are involved in nitrogen oxidation in the intestinal tract and cause an excess of urinary nitrate, the urine of conventional rats should contain more nitrate than the urine of germfree rats, assuming that dietary nitrate intakes are comparable and that nitrite synthesized in the intestine can be absorbed and stoichiometrically converted to nitrate in the bloodstream (*13*). In our first study we determined the nitrate balance of rats before and after they were removed from a germfree isolator and allowed to acquire a complex microbial flora. These rats were fed Rat Chow (Ralston Purina diet

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