Factors Affecting the Noncovalent Binding of Chlorambucil to Rabbit Immunoglobulin G

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SUMMARY

The extent to which chlorambucil binds noncovalently to rabbit immunoglobulin G (IgG) is strongly influenced by the ambient temperature, the time allowed for the reaction, and the relative concentrations of the chlorambucil and IgG. The pH and the ionic strength of the reaction mixture are much less critical. Although the pH must be alkaline, and the ionic strength must not be too great, substantial noncovalent binding occurs over a broad range of either pH or ionic strength. Under suitable conditions, up to 64.5 moles of chlorambucil were noncovalently bound per mole of IgG.

INTRODUCTION

The alkylating agent chlorambucil is able to bind firmly to serum proteins without covalently alkylating them (6–8). Recently, Ghose et al. (3, 4) presented data indicating that chlorambucil forms such noncovalent bonds with immunoglobulins and that the complex of chlorambucil with tumor-specific immunoglobulins has enhanced cytotoxic activity against a transplantable mouse tumor (3) and against a human melanoma (4). Because of the potential importance of chlorambucil-antibody complexes in the treatment of human cancer, we have developed standard procedures for obtaining such complexes free of unbound chlorambucil and quantitatively defined in terms of the number of molecules of chlorambucil bound per molecule of antibody. This report concerns our observations on the formation of the physical complex of chlorambucil with rabbit IgG. Subsequent reports will deal with the effect of physically bound chlorambucil on the specific antigen-binding capacity of rabbit IgG and the cytotoxic activity of these complexes in vivo and in vitro.

MATERIALS AND METHODS

Immunoglobulin. IgG was isolated from the serum of normal adult New Zealand White rabbits by a batch procedure. Serum was collected by cardiac puncture and dialyzed overnight against 100 volumes of 0.015 M sodium phosphate buffer at pH 6.4. After the precipitated eu-

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mixture through a Sephadex G-25 column (0.9 x 30 cm) equilibrated and eluted with 0.15 M NaCl buffered to pH 8.5 with 0.01 M Tris-HCl. This separation was carried out at 4°. The column eluate was followed at 254 nm by a UV monitor (LKB Producter AB, Stockholm, Sweden); the 1st peak to emerge, IgG with bound chlorambucil, was collected in a single fraction collector tube (about 6 ml) and chilled in an ice bath.

Quantitation. The IgG content of the eluted complex was determined by the Folin-Ciocalteu method (10); these assays were done in duplicate and then averaged. The physically bound chlorambucil could be distinguished from chlorambucil which had covalently alkylated to IgG, since the physically bound material can be separated from the protein by extraction with ethyl alcohol (7). Routinely, 5 ml of the eluted complex were quick frozen in a liquid nitrogen bath and then lyophilized. The dry residue was dissolved in 0.5 ml of cold water and 4.5 ml of cold absolute ethanol were then added. The mixture was allowed to sit in an ice bath for 5 min with occasional mixing and then the precipitated IgG, now no longer complexed with chlorambucil, was removed by centrifugation at 15,000 x g for 15 min. The protein was thus removed without diluting the freed chlorambucil below the minimal concentration required for accurate measurement. The concentration of extracted chlorambucil in the ethanolic supernatant was determined by spectrophotometry; the reading at 258 nm (7) was compared with a standard curve obtained from chlorambucil dissolved in 90% ethanol: 10% water. The presence of buffer salts in this ethanolic solvent at the concentrations encountered in these experiments did not alter the absorbance of chlorambucil at 258 nm. The method of Allen (1), as applied to chlorambucil by Linford (7), was used to correct for light scattering, although this correction was usually small and often negligible. The results of these measurements were expressed as moles of chlorambucil bound per mole of IgG.

The quantity of physically bound chlorambucil determined by this procedure includes both the active (chloro) and the inactive (hydrolyzed) forms of the compound. The portion of the total extractable chlorambucil which was in the active form was determined by a modification of the method of Allen (1), as applied to chlorambucil by Linford (7), used to correct for light scattering, although this correction was usually small and often negligible. The results of these measurements were expressed as moles of chlorambucil bound per mole of IgG.

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RESULTS AND DISCUSSION

The formation of the chlorambucil:IgG adsorption complex was examined as a function of 5 variables. The following experiments illustrate the influence of each.

**pH**. Mixtures of chlorambucil and IgG at a molar ratio of 500:1 were adjusted to various pH values and incubated for 5 min at 37°. As shown in Table I, noncovalent binding increased slightly as the pH was raised. Mixtures with pH values lower than 9.5 were not tested, since chlorambucil solutions at these concentrations began to precipitate. Mixtures with pH values greater than 11.5 also were not tested, since rabbit IgG begins to undergo irreversible denaturation at pH levels greater than 10.5 (5). Consequently, although the binding was greatest at pH 11.5, we considered pH 10.5 to be the optimum for complex formation between chlorambucil and undenatured rabbit IgG.

**Temperature**. Mixtures of chlorambucil and IgG at a molar ratio of 500:1 were adjusted to pH 11.0 and incubated for 5 min at 4° (cold room), room temperature, or 37° (water bath). Table I shows that the amount of chlorambucil bound noncovalently to IgG (ethanol-extractable chlorambucil) increased with temperature. Temperatures over 37° were not tested.

** Ionic Strength.** For this experiment, stock IgG solution in 0.15 M NaCl was dialyzed for 48 hr against water. Thus, the only NaCl present in a mixture containing this IgG and chlorambucil was produced by the slight amounts of NaOH and HCl used to dissolve the chlorambucil and to adjust the pH. Various concentrations of a stock NaCl solution were added to mixtures of chlorambucil and IgG in water to give a final molar ratio of chlorambucil to IgG of 500:1. The mixtures were adjusted to pH 10.5 and incubated for 5 min at 37°. The results are shown in Table I. Sodium chloride concentrations of 0.1 M or less had relatively little effect on adsorption. However, at higher NaCl concentrations the degree of binding began to drop off rapidly. Our standard reaction mixtures of equal volumes of IgG in 0.15 M NaCl and chlorambucil in water had a NaCl concentration of approximately 0.075 M.

**Concentration.** Chart I illustrates the effect of increasing the concentration of chlorambucil on the amount of drug physically adsorbed to IgG. The mixtures of chlorambucil and IgG were incubated for 5 min at 37° at a pH of 10.5. The total quantity of chlorambucil extractable from the IgG with ethanol increased linearly until the molar ratio of chlorambucil to IgG was 750:1. We did not determine the reason for the drop in binding found at a molar ratio of 1000:1, but it may have resulted from interference with the binding forces as a result of the very high concentration of chlorambucil or from increased alkylation of the IgG as opposed to physical adsorption.

**Time and Activity.** IgG in water was mixed with chlorambucil at a molar ratio of chlorambucil to IgG of 750:1, adjusted to pH 10.5, and incubated at 37° for increasing
**Table 1**

*Effect of pH, temperature, and ionic strength on the noncovalent binding of chlorambucil to rabbit IgG*

<table>
<thead>
<tr>
<th>Variable</th>
<th>pH</th>
<th>Temperature</th>
<th>Ionic strength</th>
<th>Moles chlorambucil bound/mole IgG</th>
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<td>0.075</td>
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<td>Ionic strength</td>
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<td>0.50</td>
<td>5.3</td>
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</table>

*a* Binding was performed for 5 min at a molar ratio of chlorambucil:IgG of 500:1.

*b* Expressed as the molarity of NaCl in the initial reaction mixture.

*c* Noncovalently bound chlorambucil only.

*d* NaCl concentration of a mixture of 1 part IgG in 0.15 M NaCl and 1 part chlorambucil in H₂O.

*e* Room temperature.

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**Chart 1.** Total chlorambucil adsorbed to rabbit IgG as a function of increasing molar ratio of chlorambucil:IgG. Chlorambucil:IgG mixtures were incubated for 5 min at 37° and pH 11.0.

**Chart 2.** Total (upper curve) and active chloro form (lower curve) of chlorambucil adsorbed to rabbit IgG as a function of time of incubation. Chlorambucil:IgG mixtures were incubated at 37° at a molar ratio of 750:1 and pH 10.5. Point marked est. was estimated, as concentration of chlorambucil was below that required for accurate assay.

The amount of chlorambucil adsorbed to IgG under these conditions was then tested for the total amount of bound chlorambucil (by spectrophotometry) and the amount of active chlorambucil (by the nitrobenzyl pyridine assay). Since chlorambucil hydrolyzes readily at 37° (9) with the release of acid, some 0.1 N NaOH was added to the mixtures incubated for 20 and 30 min to maintain the pH and prevent the chlorambucil from precipitating. As shown in Chart 2, the total amount of chlorambucil adsorbed to the IgG increased dramatically with lengthening time of incubation. After 30 min of incubation, 64.5 moles of chlorambucil were bound. This is the highest amount of binding we have observed, although incubation times greater than 30 min have not yet been tested. The amount of active chlorambucil bound with increasing time is less but still substantial. These results are not necessarily in contradiction to those of Linford (8), who found that the hydrolyzed form of chlorambucil bound much less readily to bovine serum albumin than did the chloro form. The chlorambucil we measured was all bound noncovalently to the rabbit IgG, since it was extractable by treatment with ethanol, but the inactive fraction we observed may have been formed by hydrolysis during the rather long extraction and assay procedure. This question is being examined further at present.

The mechanism of adsorption of small molecules to proteins is in general poorly understood. In the course of
many experiments with chlorambucil-IgG system, the reproducibility of binding under a given set of conditions has been generally good but not exact. Thus, while we can always obtain either high or low binding ratios by the proper choice of conditions, the variation in the ratios from one experiment to the next is somewhat greater than might be expected on the basis of chance variations in technique. Although the variables of temperature, time, pH, ionic strength and relative concentration of reactants have all been controlled, other factors, as yet unidentified, appear to have some relatively minor influence on the binding ratios. However, in spite of this problem, we have identified and standardized conditions under which high molar ratios of chlorambucil can be physically adsorbed to rabbit IgG. Moreover, we have shown that a significant amount of this bound chlorambucil is in the active form and is thus capable of subsequent alkylations, a finding which supports the hope that tumor-specific rabbit IgG may be able to deliver larger doses of active alkylating agent to tumor cells than has previously been possible (3, 4). Preliminary observations suggest that the noncovalent complex is very stable. Little or no chlorambucil, active or hydrolyzed, is dissociated from IgG during 24 hr of dialysis against water at 4°; alkylation is minimal under these conditions. At 37°, there is also no apparent dissociation but alkylation occurs readily.

Some obvious questions remain to be answered. How much chlorambucil (active and/or inactive) can be adsorbed to a molecule of rabbit IgG antibody without harming the antibody specificity and antigen-binding capacity? Is a complex of chlorambucil and appropriate tumor-specific antibody more toxic to tumor cells than is the equivalent amount of antibody or chlorambucil alone? Can it be maintained longer in an active form if frozen or freeze-dried? Data pertinent to these questions will be reported in a subsequent paper.

REFERENCES

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