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Affinity Labeling of Equine Anti-β-lactoside Antibodies*

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ABSTRACT: Equine anti-Lac antibodies of two varieties, γG and γG(T)[2] isolated from a single horse, have been labeled specifically with OD-Lac as affinity labeling reagent. The reaction conditions were the same as those employed in affinity labeling of pooled rabbit anti-Lac antibodies with the same reagent. Azo spectra of H and L chains from the labeled equine antibodies of both varieties are qualitatively indistinguishable. However, these spectra show that the residues selectively modified by affinity labeling of the equine anti-Lac antibodies differ distinctly from those labeled on both H and L chains of the rabbit antibodies. The possible genetic or evolutionary significance of these characteristic distinctions is not yet established.

Affinity labeling studies with rabbit anti-Lac[1] and anti-Gal antibodies point to particular chemical features shared by antibodies of similar (saccharide) specificity which distinguish them from antibodies of grossly different (benzenoid) specificity (Wofsy *et al.*, 1967). The results produced in affinity labeling of antibenzenoid hapten antibodies (namely, the selective modification at active sites of tyrosine residues on both heavy (H) and light (L) chains) have been shown in the case of anti-dinitrophenyl antibodies to hold for all of four species thus far studied (A. H. Good, Z. Ovary, and S. J. Singer, unpublished data). In the present study equine 7S anti-Lac antibodies of two structural varieties, γG and γG(T)[2] (Rockey *et al.*, 1964; Klinman *et al.*, 1964, 1965), have been labeled with the same reagent, OD-Lac, and under the same conditions as were employed in affinity labeling of rabbit anti-Lac antibodies (Wofsy *et al.*, 1967).

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[1] Abbreviations used: anti-Lac and anti-Gal, antibodies specific for the respective haptenic groups *p*-azophenyl β-lactoside and *p*-azophenyl β-galactoside; OD-Lac, the reagent *o*-diazoniumphenyl β-lactoside; SDS, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene.

[2] This was formerly designated γA on the basis of its carbohydrate content and behavior in immunoelectrophoresis. Recent observations have indicated its identity with the T component of equine antiserum as judged by its antigenic identity with similarly prepared diphtheria antitoxin and tetanus antitoxin (R. Genes, N. R. Klinman, R. Hirsh, and F. Karush, unpublished data). In view of the antigenic similarity between γG-immunoglobulin and the T component (Weir and Porter, 1966) and the extensive homology of the C-terminal sequences o their heavy chains (Weir *et al.*, 1966), the designation γG(T), suggested by Weir *et al.* (1966) will be used instead of γA.

Materials and Methods

Preparation of Antigens and Antibodies. The preparation of antigens has been previously described (Karush, 1957). The antibodies used in this study, as well as the immunization and purification procedures by which they were obtained, have been presented in detail elsewhere (Klinman *et al.*, 1965, 1966). The particular antibody preparations employed were isolated from a pool of serum taken from the same horse during the 12th to 42nd week of a second course of immunization. Injections at biweekly or monthly intervals consisted of 40–80 mg of antigen dispersed in incomplete Freund's adjuvant or alum precipitated. The immunizing antigen during this period was the azoconjugate of the Lac haptenic group and hemocyanin, *Limulus polyphemus.*

After the multiple types of anti-Lac antibodies were coprecipitated from the serum and freed from antigen as before, γG and γG(T) immunoglobulins were separated by placing purified antibody (10 mg/ml) on a DEAE-cellulose column (Whatman DE 52 microgranular 1 mequiv/g) equilibrated to 0.01 M sodium phosphate buffer (pH 7.85) and increasing the ionic strength by stepwise addition of sodium chloride first to a concentration of 0.04 M and then to 0.3 M. The purity of the γG and γG(T) preparations was established as before on the basis of their characteristic immunoelectrophoretic patterns developed with goat antisera to heavy chains of equine immunoglobulins.

The binding of tritium-labeled *p*-(*p'*-dimethylaminobenzene)azophenyl β-lactoside by γG and γG(T) antibodies was measured by equilibrium dialysis by a previously described technique (Karush, 1957). Free-dye concentrations were obtained by scintillation counting in Bray's (1960) phosphor solution. The γG antibody preparation bound hapten at 25° with a K_0 of 8.2×10^6 M^{-1}; the γG(T) showed a K_0 of 1.8×10^7 M^{-1}. More extensive analysis of the binding properties of these antibodies is presented elsewhere (Karush and Sela, 1967).

Reagents and Affinity Labeling Procedures. The reagents, buffers, labeling procedures, work-up of labeled proteins, and spectral assay methods were the same as those employed in affinity labeling of rabbit anti-Lac antibodies with OD-Lac (Wofsy *et al.*, 1967). Where the H and L chains of labeled antibodies were examined, the antibodies were mildly reduced and alkylated and then, following labeling, the chains were separated on Sephadex G-100 in 1 N propionic acid exactly as in the rabbit studies.

Spectral determinations were made with a Zeiss PMQ II spectrophotometer. Protein concentrations were obtained from values of $OD_{280\,m\mu}$ in 0.5% SDS–0.02 M sodium phosphate buffer (pH 6.2) based on the following extinction coefficients for 1% protein solutions: for γG and γG(T) antibodies, $\epsilon^{1\%}_{280\,m\mu}$ 14.7; for both types of H chains, $\epsilon^{1\%}_{280\,m\mu}$ 15.4; and for L chains, $\epsilon^{1\%}_{280\,m\mu}$ 14.0 (Rockey, 1967).

Tritiated *o*-aminophenyl β-lactoside was synthesized by the general methods described in Wofsy *et al.* (1967) starting with the condensation of acetobromolactose with tritiated *o*-nitrophenol (New England Nuclear).

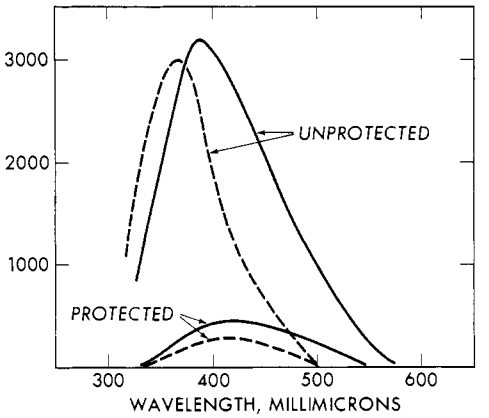

FIGURE 1: Azo spectra at pH 6.2 (– – –) and in 0.15 N NaOH (——) of equine γG(T) anti-Lac antibodies reacted with OD-Lac in presence (protected) and absence (unprotected) of *p*-nitrophenyl β-lactoside. Extinction coefficients (ϵ) based on 150,000 g/l.

Condensation products were separated by thin layer chromatography (tlc) (Brinkman Instruments, Inc., type silica gel F) developed with benzene–ethyl acetate (2:1, v/v); for *o*-nitrophenyl-^{3}H heptacetyl-β-lactoside, R_F .5. Deacetylation of the heptacetyl derivative with dilute sodium methylate in methanol and purification by descending paper chromatography (S & S orange) developed with 1-butanol–pyridine–water (63:35:45) afforded *o*-nitrophenyl-^{3}H β-lactoside (R_F 0.6). Catalytic reduction and paper chromatography (same system as above) yielded *o*-aminophenyl-^{3}H β-lactoside (R_F 0.5). All R_F values were verified with authentic compounds previously synthesized (Wofsy *et al.*, 1967).

Tritiated protein solutions were desalted on Sephadex G-25 in 10^{-3} M acetic acid. Aliquots were lyophilized in Wheaton vials and solubilized with 0.4 ml of 0.6 N NCS reagent (Nuclear-Chicago). After addition of liquid scintillation fluid (toluene–PPO–POPOP), the solutions were counted in a Nuclear-Chicago liquid scintillation counter (Mark I). Counting efficiency was determined by the internal standard method, with tritiated toluene (New England Nuclear) as standard.

Results

Two solutions of 2.0×10^{-5} M equine γG(T) anti-Lac antibodies, one of which was 10^{-3} M in *p*-nitrophenyl β-lactoside, were reacted with 1.1×10^{-5} M OD-Lac in 0.12 M sodium borate–0.17 M saline (pH 8), 5°, for 1 hr. In Figure 1, the azo spectra of the reacted antibodies, after purification and dissolution in 0.5% SDS, are compared. Modification of the antibodies reacted in the presence of the protector, *p*-nitrophenyl β-lactoside, was slight. The unprotected antibodies show azo maxima at 370–375 mμ at pH 6.2, and at 390 mμ in 0.15 N NaOH; at the maxima, azo absorbance is seven to ten times greater for the unprotected than for the protected antibodies.

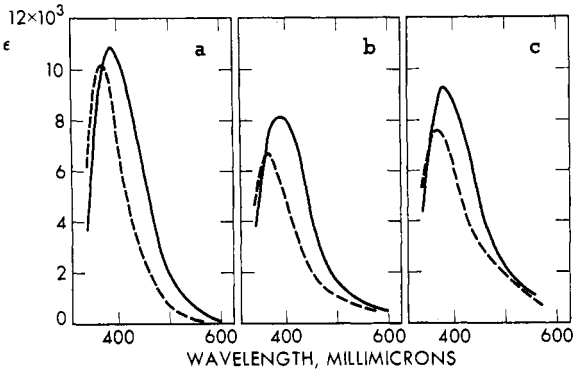

FIGURE 2: Azo spectra at pH 6.2 (- - -) and in 0.15 N NaOH (——) of OD-Lac-labeled equine γG(T) anti-Lac antibodies and their polypeptide chains. (a) Mildly reduced and alkylated antibodies, undissociated; (b) H chains; (c) L chains. ε values based on 150,000 g/l.

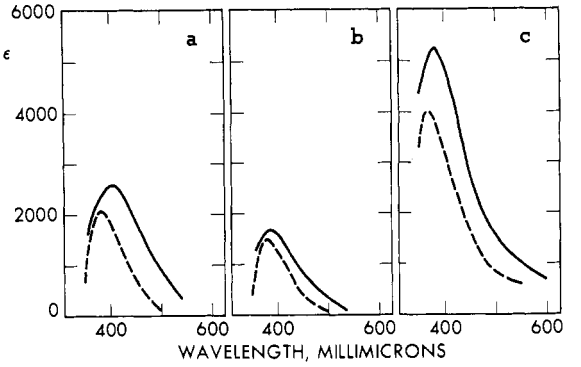

FIGURE 3: Azo spectra at pH 6.2 (- - -) and in 0.15 N NaOH (——) of OD-Lac-labeled equine γG anti-Lac antibodies and their polypeptide chains. (a) Mildly reduced and alkylated antibodies, undissociated; (b) H chains; (c) L chains. ε values based on 150,000 g/l.

Mildly reduced and alkylated preparations of equine γG(T) and γG anti-Lac antibodies, each 2.0×10^{-5} M in protein concentration, were reacted with 2.2×10^{-5} M OD-Lac at pH 8, 5°, for 50 min. Another aliquot of each antibody solution was made 10^{-3} M in *p*-nitrophenyl β-lactoside and then reacted with OD-Lac in exactly the same manner as the unprotected antibodies. Samples from each antibody solution were separated into H and L chain fractions, and azo difference spectra were determined by subtracting the absorbance of protected protein from equivalent unprotected protein. Figure 2 shows the azo spectra at pH 6.2 and in 0.15 N NaOH of 0.5% SDS solutions of the unfractionated preparation, H chains, and L chains of the labeled γG(T) anti-Lac antibodies. Figure 3 shows the corresponding spectra for the labeled γG anti-Lac antibodies. While there are differences in the extent of labeling, the spectral characteristics are very similar in all cases: at pH 6.2, the maxima are between 365 and 385 mμ; and in 0.15 N NaOH, the maxima are between 380 and 405 mμ.

Two aliquots of γG anti-Lac antibodies, one of which was 10^{-3} M in *p*-nitrophenyl β-lactoside, were reacted with OD-Lac-^{3}H at pH 8, 5°, for 30 min. After work-up, the unprotected sample contained 3015 dpm/mg, whereas the protected sample had 288 dpm/mg.

Discussion

Affinity labeling of equine γG and γG(T) anti-Lac antibodies has been accomplished with the same reagent, OD-Lac, and under the same conditions as were successfully applied with rabbit 7S anti-Lac antibodies. The specificity of labeling under these conditions has been demonstrated by the marked protection observed in the presence of *p*-nitrophenyl β-lactoside. Only about one-tenth as much reaction occurs with protected as opposed to unprotected antibodies, as shown by comparative azo absorbance (γG(T), Figure 1) or radioactivity (γG antibodies labeled with OD-Lac-^{3}H).

As in all previous affinity labeling studies with anti-hapten antibodies (Singer and Doolittle, 1966; Wofsy *et al.*, 1967), label is found on both H and L chains. The azo spectra of labeled equine γG and γG(T) anti-Lac antibodies, and of their respective H and L chains, appear qualitatively indistinguishable. The absorbance maxima are within a narrow spectral range, 365–385 (at pH 6.2) and 380–405 mμ (in 0.15 N NaOH). There is a spectral resemblance to the monoazo derivative of *N*-acetylhistidine and OD-Lac, which shows a peak at 375–380 (at pH 6.2) and 405–410 mμ (in 0.15 N NaOH) (Figure 2 in Wofsy *et al.*, 1967). It is not possible to rule out modification of lysine residues, since the model compound of monoazolysine, the presumed product of such a putative affinity labeling reaction, has not been synthesized; however, it is unlikely that an azolysine derivative would be stable under the conditions employed for chain separation, work-up, and spectral examination of the labeled antibodies.

Assuming that the predominant product of the labeling reaction is the same for both chains of both antibodies and taking into account the molecular weights of H (50,000) and L (23,000), it may be estimated that the molar ratio of labeled H:L chains is about two for the γG(T) (Figure 2) and about one for the γG antibodies (Figure 3). While the azo extinctions of the H chains of labeled γG anti-Lac are low, the azo difference spectra in Figure 3 cannot be owing to a nonspecific reaction since they were determined by subtracting the absorbance of equivalent protected protein. Nor can the H chain azo spectrum of Figure 3b be attributed to possible contamination with L chains; for if only L chains were labeled, their contribution would be insufficient to account for the azo extinctions of the unfractionated antibody (Figure 3a).

Of particular interest is the fact that the spectral patterns obtained in the affinity labeling experiments with these equine anti-Lac antibodies are distinctly different from those resulting when rabbit anti-Lac antibodies are labeled with OD-Lac in an identical manner. The azo spectrum characteristic of H and L chains of both

varieties of the equine anti-Lac antibodies is readily distinguished from that of azotyrosine, which is found on labeled rabbit anti-Lac H chains, or of the unidentified derivative with an absorbance maximum at 510 mμ which is found on labeled rabbit anti-Lac L chains (Figures 4 and 5 in Wofsy *et al.*, 1967). In view of the possibility that histidine is the residue selectively labeled on both chains of both classes of the equine anti-Lac antibodies, it may be relevant to consider data on the comparative histidine content of the chains of equine and rabbit anti-Lac antibodies (Rockey, 1967; Koshland *et al.*, 1966). For the H chains, per 50,000 g, there are 9.8–10.4 histidine residues in equine γG and 12.5 in equine γG(T), while there are 6.8 in the rabbit anit-Lac antibodies; for the L chains, per 23,000 g, there are 2.6 histidine residues in the equine as compared to 1.4 in the rabbit anti-Lac antibodies.

The fact that antibody molecules with specific affinity for a particular hapten may differ in chemical composition at or in the immediate vicinity of active sites has been suggested by studies such as those on the heterogeneity of rabbit antidinitrophenyl antibodies by Eisen and Siskind (1964) and is shown directly in the results of Doolittle and Singer (1965) on the heterogeneity of peptides derived from the active sites of affinity labeled rabbit anti-DNP antibodies. Yet even within such heterogeneous antihapten antibody populations, discrete affinity labeling patterns, as well as the results of active-site labeling by other techniques (Koshland *et al.*, 1959; Pressman and Roholt, 1961), reveal distinctive features that relate many antibody molecules comprising a broadly defined " common specificity."

It is not yet possible to establish the significance of the striking chemical difference revealed in comparing the affinity labeling results obtained in the case of anti-Lac antibodies from a single horse with those observed in studies on rabbit anti-Lac antibodies from pooled antisera. Other horses are currently being immunized against hemocyanin azophenyl β-lactoside so that subsequently we may determine whether the differences reported in these studies are characteristic of the two species or reflect distinctions in the antibody response of individual animals. If two animals (or two species) may be distinguished from each other by the distinctive chemical composition of the active sites of antibodies with a common specificity, this might imply the presence of genetically determined variants (or of species specific evolutionary alternatives) in the amino acid sequences of those portions of the H and L chains that define antibody specificity.

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