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Immunology

# Biological significance of carbohydrate chains on monoclonal antibodies

**(tunicamycin/Fc receptor/complement/protein A/immunocomplex)**

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**ABSTRACT** **We have prepared monoclonal hapten-specific mouse IgG2b antibodies depleted of asparagine-linked carbohydrate chains by treating the hybridoma cells with tunicamycin. The carbohydrate-deficient antibodies behaved in an identical manner to the normal antibodies with regard to fine antigen-binding reactivity (a Fab fragment feature) and protein A binding capacity [a feature requiring integrity at the $C_H2$ and $C_H3$ domain-interaction regions in the constant region of the heavy chain ($C_H$)]. However, they lost the ability to activate complement, to bind to Fc receptors on macrophages, and to induce antibody-dependent cellular cytotoxicity. Furthermore, antigen–antibody complexes produced from such carbohydrate-deficient antibodies failed to be eliminated rapidly from the circulation. We conclude that removal of carbohydrate chains from IgG molecules may have a profound and highly select impact on the biological activity to these antibodies.**

Carbohydrates have been indicated to be of significant importance in secretion of Ig molecules (1), recognition of polymeric antigens (2), and creation of IgG–IgG complexes (3). Likewise complement fixation, opsonic activity, and the binding to Fc receptors of rabbit IgG antibodies may require carbohydrate moieties (4, 5). We have taken advantage of the availability of monoclonal antibody-producing hybridoma cells to obtain homogeneous antibodies with defined specificity. As carbohydrates on IgG and IgM are asparagine-linked (6–8), tunicamycin (Tm) blocking of the dolichol-dependent, asparagine-linked glycosylation pathway (9) can be used *in vitro* to obtain, through biosynthesis, carbohydrate-deficient Ig molecules (1). Our comparison of monoclonal IgG2b antibodies with or without carbohydrate chains demonstrates that the absence of carbohydrates will lead to an inability of the antibodies to function in antibody-dependent cellular cytotoxicity (ADCC), to interact with Fc receptors on macrophages, to activate complement, and to be eliminated rapidly from the circulation only when complexed with antigens. On the other hand, no reduction in protein A binding capacity or in affinity of the antibodies was noted upon carbohydrate depletion. The implications of these findings will be discussed.

## MATERIALS AND METHODS

**Cells and Reagents.** Mouse anti-trinitrophenyl (anti-TNP) antibody-producing hybridoma cells, GKH-1 GORK IgG2b, Hy 1.2 IgG2a, Hy2.15Agl IgG1, and Sp6 IgM, were gifts from Georges Köhler (Basel Institute for Immunology). The murine macrophage cell line M1 was provided by Yasou Ichikawa (Kyoto University Chest Disease Research Institute). Cells were kept in Dulbecco's modified Eagle's medium (DME medium) containing 10% heat-inactivated fetal calf serum (GIBCO), 100 $\mu$g of streptomycin per ml, 100 units of penicillin per ml, and 10 mM Hepes. Peritoneal cells were obtained from untreated 4-mo-old CBA/H mice by lavage. Spleen cells were prepared by gently teasing the spleen. Sheep erythrocytes (SRBC) stored in Alsever's solution at 4°C were used.

Tm was donated by Akira Takatsuki (Tokyo University, Faculty of Agriculture). Bovine serum albumin fraction V (Sigma); Sephadex G-25 and G-200, Sepharose 4B, CNBr-activated Sepharose 4B, protein A-Sepharose CL-4B, and protein A from *Staphylococcus aureus* (Pharmacia); L-[4,5-$^3$H]leucine (136 Ci/mmol; 1 Ci = 37 GBq), D-[U-$^{14}$C]glucosamine (277 mCi/mmol), $Na_2{}^{51}CrO_4$ (150–250 $\mu$Ci/$\mu$g of Cr), and $Na^{125}I$, carrier free (Radiochemical Centre), were used.

**Biosynthetic Radiolabeling of Ig and TNP Binding Assay.** Hybridoma cells were put in the labeling medium (F10 medium without L-leucine containing 15% fetal calf serum pretreated with TNP-coated SRBC, 4 mM of L-glutamine, 100 $\mu$g of streptomycin per ml, 100 units of penicillin per ml, and 10 mM Hepes) at a cell density of $1 \times 10^6$ cells per ml. In some experiments (see Fig. 1), hybridoma cells (0.2 ml) were cultured in microplates (Falcon 3040) with Tm for 1 hr. [$^3$H]Leucine and [$^{14}$C]glucosamine (1 $\mu$Ci each) were then added to each sample. After 18 hr, the microplates were centrifuged for 30 min at $1,000 \times g$ to collect the supernatant. In other experiments (see Table 1), hybridoma cells precultured with Tm in DME medium for 6 hr, followed by washing in Hanks' balanced salt solution, were cultured with [$^3$H]leucine and [$^{14}$C]glucosamine (5 $\mu$Ci/ml each) in a tissue culture flask (Nunc) in the renewed presence of Tm for 18 hr. Culture supernatants were collected after centrifugation at $4,000 \times g$ for 30 min and used as antibodies.

Incorporation of [$^3$H]leucine and [$^{14}$C]glucosamine into anti-TNP antibodies was analyzed by binding to TNP-SRBC prepared as described (10). Each sample (0.1 ml) was mixed with $4 \times 10^7$ cells of TNP-SRBC or SRBC in 0.1 ml of 0.1% gelatin/Veronal buffer containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (pH 7.4) ($GVB^{2+}$) in a microtiter plate (Cooke, UK). After incubation for 1 hr at 4°C, TNP-SRBC or SRBC were washed in $GVB^{2+}$ dissolved in 0.2 ml of 1% $NaDodSO_4$ solution, and transferred to 2 ml of a 2:1 (vol/vol) toluene/Triton X-100-based scintillation solution. Ig specifically bound to TNP was determined by subtracting the radioactivity bound to SRBC, which was always <10% of that of TNP-SRBC. Furthermore, adding TNP-bovine serum albumin (TNP-albumin) in excess did inhibit >98% of the binding of Ig to TNP-SRBC (data not included). The antibody level of each sample also was measured by hemagglutination (11) of TNP-SRBC.

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Abbreviations: Tm, tunicamycin; SRBC, sheep erythrocytes; TNP, trinitrophenyl; ADCC, antibody-dependent cellular cytotoxicity; TNP-albumin, TNP-bovine serum albumin; $C_H$, constant region of heavy chain.

**Cytotoxicity Assays.** Cytotoxicity was determined by release of $^{51}Cr$ from TNP-SRBC targets (12). In complement-mediated lysis, $10^5$ targets were incubated with antibodies in 0.2 ml of $GVB^{2+}$ containing guinea pig complement at 37°C for 1 hr. In ADCC $10^5$ M1 cells or $10^6$ spleen cells as effectors were incubated with $10^5$ targets in 0.2 ml of DME medium with antibodies at 37°C for 18 hr. The percentage of chromium released was calculated from the formula: [(ER − CR)/(MR − CR)] × 100, where ER is experimental release, CR is control release in the presence of complement or effector cells without antibodies, and MR is maximum release obtained by the incubation with 0.05% of Nonidet P-40 solution.

**IG-Coated TNP-SRBC and Fc Rosettes.** Ig-coated TNP-SRBC were prepared (13) by using control or carbohydrate-depleted IgG2b antibodies in the same subhemagglutination titer. TNP-SRBC treated with labeling medium only were used as control TNP-SRBC. To assay Fc rosettes (14), mouse peritoneal cells or M1 cells were used.

**Purification of IgG2b and $^{125}$I-Labeling of Proteins.** IgG2b antibodies in the culture supernatant of GORK hybridoma cells cultured in the presence or absence of Tm were purified by using protein A-Sepharose CL-4B (15). Some IgG2b antibodies were further purified on TNP-albumin-Sepharose 4B (16, 17).

Control and carbohydrate-depleted IgG2b purified by protein A, followed by TNP-albumin immunoabsorbent elution (100 μg each), were iodinated with 1 mCi of $Na^{125}I$ (18). TNP-albumin and protein A were likewise iodinated.

**Polyacrylamide Gel Electrophoresis.** Internally radiolabeled, protein A-bound, and eluted IgG2b was analyzed by 7% $NaDodSO_4$/polyacrylamide gel electrophoresis as described by Weber and Osborn (19), after reduction by heating in an 80°C water bath for 5 min in the loading buffer (1% $NaDodSO_4$/0.01 M phosphate buffer, pH 7.0/5% 2-mercaptoethanol). After electrophoresis the disk gels were frozen and sliced into 1-mm slices. The radioactivity of [$^3$H]leucine and [$^{14}$C]glucosamine in each slice was determined after elution into 0.3 ml of 1% $NaDodSO_4$ by incubation on a shaker at 37°C overnight and transfer to a scintillation solution.

**Preparation of Immunocomplexes and Elimination Studies *in Vivo*.** $^{125}$I-Labeled TNP-albumin (6 μg) was mixed with an excess of protein A-purified, control or carbohydrate-depleted IgG2b (1 mg) in 1 ml of 0.14 M NaCl/0.01 M sodium phosphate, pH 7.2 ($P_i$/NaCl) at 37°C for 30 min. Each mixture was gel-filtered on a 10-ml Sephadex G-200 column. The excluded pool (>97% of total $^{125}$I-labeled TNP-albumin) was analyzed in a 10-ml Sepharose 4B column. The general size distribution of either type of immunocomplex was found to be quite similar, admittedly with still some 50% of immunocomplexes being in the excluded fraction. They were used as control or carbohydrate-depleted immunocomplexes.

Elimination studies of immunocomplexes from the circulation were performed on 4-mo-old CBA/H mice as described (20). $^{125}$I cpm in serum samples (0.1 ml each) and in their precipitates from 50% ammonium sulfate were determined.

## RESULTS

**Depletion of Carbohydrate Chains on Ig Molecules.** The TNP-specific hybridoma cells were screened for sensitivity to Tm-induced inhibition of secretion in relation to carbohydrate depletion. The IgG2b-producing GORK line allowed secretion of Ig molecules to a significant extent even when carbohydrate depletion approached 100% (Fig. 1). In contrast, the IgM-producing hybridoma was more sensitive to inhibition of secretion than to removal of carbohydrate moieties. IgG2a and IgG1 hybridoma cells behaved like the IgM hybridoma. Thus, the GORK cell line was chosen for further studies.

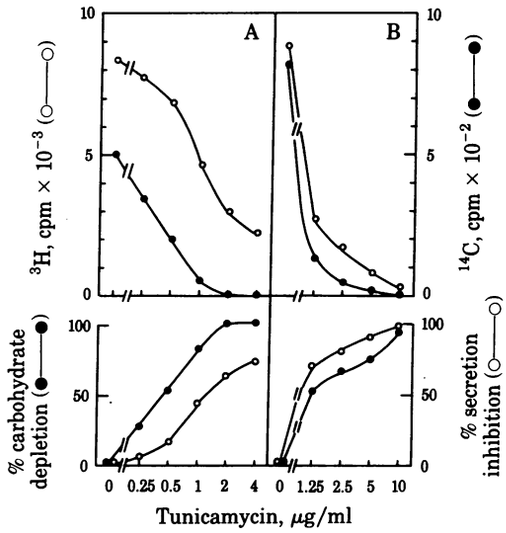


FIG. 1. [$^3$H]Leucine and [$^{14}$C]glucosamine incorporated into anti-TNP antibodies. (*A*) GORK IgG2b. (*B*) Sp6 IgM. Cells were cultured with the indicated concentration of Tm for 1 hr prior to biosynthetically labeling with [$^3$H]leucine and [$^{14}$C]glucosamine. After 18 hr, radioactivity of secreted antibodies was analyzed by the TNP binding assay. Percentages of secretion-inhibition and carbohydrate-depletion were calculated from the following formulas, respectively:

$$\left(1 - \frac{^3\text{H cpm with Tm}}{^3\text{H cpm without Tm}}\right) \times 100;$$

$$\left(1 - \frac{^{14}\text{C cpm}/^3\text{H cpm with Tm}}{^{14}\text{C cpm}/^3\text{H cpm without Tm}}\right) \times 100.$$

Each point represents the mean of duplicate samples.

We next analyzed the time required after Tm administration to allow the intracytoplasmic pool of antibody molecules to turn over. When GORK cells preincubated with [$^3$H]leucine were cultured, >99% of the intracytoplasmic pool of IgG2b antibodies ($^3$H-positive) was secreted during the first 6 hr, regardless of the presence of Tm. Accordingly we chose to preincubate GORK cells with Tm for 6 hr, followed by washing before radiolabeling, as shown in Table 1. IgG2b antibodies obtained from such Tm-treated GORK cells were dramatically reduced in [$^{14}$C]glucosamine content while retaining normal hemagglutination titers when compared at the same protein ([$^3$H]leucine)

Table 1. Anti-TNP monoclonal IgG2b antibodies depleted of carbohydrate contents in the culture supernatant of GORK hybridoma cells

| Batch | Tm, μg/ml | Incorporation into antibodies bound to TNP-SRBC, cpm: [$^{14}$C]Glucosamine | Incorporation into antibodies bound to TNP-SRBC, cpm: [$^3$H]Leucine | % carbohydrate depletion* | Hemagglutination of TNP-SRBC, dilution titer |
|---|---|---|---|---|---|
| A | 0 | 965 ± 130† | 7,780 ± 220 | | $3 \times 2^5$ |
| | 1.5 | 50 ± 10 | 3,055 ± 185 | 87.1 | $3 \times 2^4$ |
| B‡ | 0 | 3,035 ± 260 | 4,405 ± 305 | | $3 \times 2^5$ |
| | 1.0 | 148 ± 9 | 1,740 ± 105 | 87.7 | $3 \times 2^4$ |
| C | 0 | 362 ± 50 | 7,708 ± 194 | | $3 \times 2^5$ |
| | 1.0 | 53 ± 16 | 4,700 ± 78 | 80.9 | $3 \times 2^4$ |

* Calculated according to the formula shown in Fig. 1.
† The mean cpm of triplicate samples ± SD.
‡ DME medium was used instead of F10 medium.

concentration. Moreover, by using $NaDodSO_4$/polyacrylamide gel electrophoresis, protein A-bound and eluted Ig molecules (batch C, Table 1) were shown to be highly depleted of carbohydrate contents normally linked to the heavy chains of IgG2b molecules (not shown). In addition, no significant amount of split peptide fragments of carbohydrate-depleted IgG2b molecules was observed.

**Impact of Carbohydrate Deficiency in IgG2b Antibodies.** *Failure to influence fine antigen-binding capacity or protein A binding ability.* The Farr precipitation assay will detect differences in avidity (21). Absence of carbohydrates had no detectable effect on the capacity of IgG2b antibodies to react with TNP (Fig. 2*A*). Protein A from *Staphylococcus aureus* has the select ability to react with a region involving the $C_H2$ and $C_H3$ domains of the heavy chain constant regions ($C_H$) of most mammalian IgG classes (23, 24). Normal and carbohydrate-deficient IgG2b antibodies displayed identical protein A binding capacity (Fig. 2*B*). $C_H2$ or $C_H3$ domains do not alone bind to protein A (25). The interaction requires an intact $C_H2$-$C_H3$ interface (24). Thus, we can conclude that absence of carbohydrates has no general effect on conformation of the Fc region of IgG2b antibodies as measured by protein A.

*Reduction in Fc receptor binding ADCC ability.* Fc receptors on mouse macrophages recognize sites present at the $C_H2$ domains of mouse IgG2b molecules (14). Normal and carbohydrate-deficient IgG2b anti-TNP antibodies were used for coating TNP-SRBC and subsequent testing for rosette formation with peritoneal macrophages or the macrophage cell line M1 (26). Carbohydrate-deficient antibodies largely failed to induce such rosette formation (percentage reduction, 91% for M1 cells and 90% for peritoneal macrophages). Likewise, when the ability of $^{125}$I-labeled TNP-albumin–anti-TNP immunocomplexes to bind to these cells was analyzed, no significant binding was noted in the complexes made up from carbohydrate-deficient antibodies (Fig. 3*A*). No differences in the formation of both types of antibodies were noted when measured in another assay, the Farr system (Fig. 3*B*). Furthermore, in ADCC with either M1 or whole spleen cells as effector cells (Fig. 4), antibodies lacking carbohydrates were virtually inactive as inducing agents ($^1/_{16}$ to $^1/_8$ as efficient as the corresponding normal antibody molecules). Thus, the data obtained would indicate that the carbohydrate chains present in the IgG2b $C_H2$ domains are fundamental in creating a proper recognition between IgG and the macrophage Fc receptor.

*Reduction in complement activation capacity.* Our studies had shown that binding to TNP-coated erythrocytes was identical for normal and carbohydrate-deficient antibodies (Table 1). The ability of the latter to lyse TNP-SRBC was, however, significantly impaired in complement-mediated lysis (Fig. 5) (≈75% reduction). To exclude the possibility that natural antibodies present in guinea pig serum against murine IgG2b carbohydrates may cause the observed differences, we also used guinea pig complement pretreated with a normal GORK IgG2b immunosorbent. The results demonstrated, however, an even greater reduction in complement-activating capacity of the carbohydrate-deficient antibodies (Fig. 5). The binding site for the C1 complement factor is localized to the $C_H2$ domain of IgG (27). The reduction in complement-inducing ability was in the same order of magnitude as the carbohydrate depletion (see

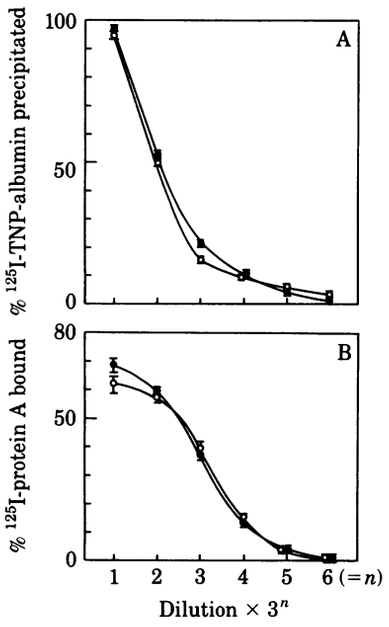


FIG. 2. Antigen-binding capacity of IgG2b antibodies (*A*) and protein A binding capacity (*B*). The antibodies (not purified; batch A of Table 1)—control (○) and carbohydrate-depleted (●)—were used in the same hemagglutination in both experiments. (*A*) Based on the Farr technique (22), $^{125}$I-labeled TNP-albumin ($^{125}$I-TNP-albumin) (0.14 μg; $5 \times 10^4$ cpm) was incubated with the antibodies serially diluted in 0.5 ml of the labeling medium at 4°C overnight, and then the 50% ammonium sulfate-precipitable radioactivity of $^{125}$I-TNP-albumin was assayed. Ammonium sulfate precipitation of $^{125}$I-TNP-albumin alone in the labeling medium did not exceed 5% of trichloroacetic acid-precipitable radioactivity. (*B*) $^{125}$I-Labeled protein A ($^{125}$I-protein A) (0.1 μg; $5 \times 10^5$ cpm) was incubated with IgG2b-coated TNP-SRBC or noncoated TNP-SRBC serially diluted in 50 μl of 1% ovalbumin/$P_i$/NaCl (initial cell number, $5 \times 10^6$). After incubation at 4°C for 1 hr, each sample was washed in 1% ovalbumin/$P_i$/NaCl, and then the radioactivity specifically bound to IgG2b-coated TNP-SRBC was determined by subtracting the amount of $^{125}$I-protein A bound to noncoated TNP-SRBC. Each point represents the mean of triplicate samples ± SD, expressed as the percentage of 10% trichloroacetic acid-precipitable radioactivity of administered $^{125}$I-TNP-albumin or $^{125}$I-protein A.

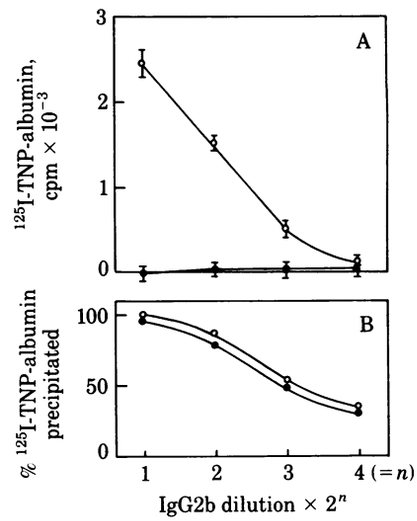


FIG. 3. Immunocomplex binding of M1 cells. Immunocomplexes were prepared in the mixture of $^{125}$I-labeled TNP-albumin ($^{125}$I-TNP-albumin) (0.3 μg; $1 \times 10^5$ cpm) and 1 ml of the antibodies (not purified; batch A of Table 1)—control (○) or carbohydrate-depleted (●)—which had been adjusted to the same hemagglutination and serially diluted with the labeling medium. (*A*) M1 cells ($10^5$) were incubated in 0.1 ml of the mixture of immunocomplexes at 4°C for 1 hr. After the cells were washed, the radioactivity of $^{125}$I-TNP-albumin bound to M1 cells was assayed. Each point represents the mean cpm ± SD from triplicate samples. (*B*) The relative amount of immunocomplexes in the mixture was tested by the Farr technique (see Fig. 2*A*). Each point represents the mean from duplicate samples.

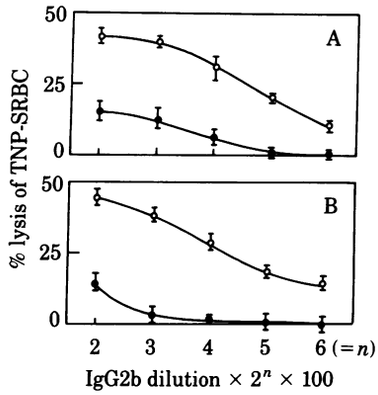


FIG. 4. ADCC of M1 cells (*A*) and spleen cells (*B*) against $^{51}$Cr-labeled TNP-SRBC. IgG2b antibodies—control (○) and carbohydrate-depleted (●)—which had been purified from batch B of Table 1 by a protein A column, were used in the same hemagglutination. Each point represents the mean of triplicate samples ± SD. Control release of $^{51}$Cr-labeled TNP-SRBC was 21% of the incorporated count; maximum release was 96%.

Table 1). Thus, absence of the carbohydrate chains normally localized to $C_H2$ domains may inactivate the C1 site as to its inducing/binding ability.

*Failure of immunocomplexes to display a rapid elimination* in vivo. We next studied the capacity of carbohydrate-deficient antibodies *in vivo* to cause elimination of immunocomplexes from the circulation. $^{125}$I-Labeled TNP-albumin was used to create complexes with either normal or carbohydrate-deficient antibody molecules. The rate of elimination of such immunocomplexes from the circulation did differ dramatically, at least initially; whereas normal antibody–antigen complexes were rapidly eliminated, carbohydrate-deficient antibody–antigen complexes were eliminated at the same rate as antigen alone (Fig. 6 *Left*). This difference was further emphasized by the finding that the amount of antigen still in combination with antibody in serum after 30 and 90 min was only 67.5% and 35.8% for the normal antibody–antigen complexes in contrast to 93.0% and 68.8% for the carbohydrate-deficient complexes (analyzed by radioactivity precipitable in 50% ammonium sulfate solution). On the other hand, carbohydrate-deficient antibodies alone were almost eliminated in the same manner as control Ig molecules (Fig. 6 *Right*).

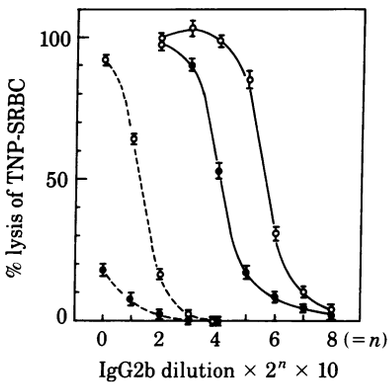


FIG. 5. Complement-mediated cytotoxicity of IgG2b antibodies against $^{51}$Cr-labeled TNP-SRBC. Both antibodies (not purified; batch A of Table 1)—control (○) and carbohydrate-depleted (●)—were used in the same hemagglutination. The antibodies in serial dilutions were incubated with $^{51}$Cr-labeled TNP-SRBC in the presence of guinea pig complement, which had been absorbed with TNP-SRBC (——) or control IgG2b-coated TNP-SRBC (----) in $P_i$/NaCl at 4°C. Each point represents the mean of triplicate samples ± SD. Control release of $^{51}$Cr-labeled TNP-SRBC was 9% of the incorporated count; maximum release was 95%.

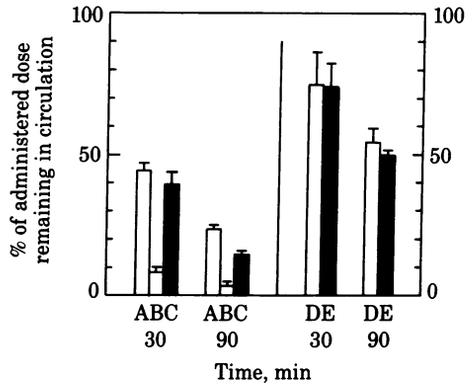


FIG. 6. Elimination of IgG2b complexes (*Left*) and IgG2b antibodies alone (*Right*). Lanes: A, antigen alone [$^{125}$I-labeled TNP-albumin ($^{125}$I-TNP-albumin)]; B, control IgG2b complexes ($^{125}$I-TNP-albumin + control IgG2b); C, carbohydrate-depleted IgG2b complexes ($^{125}$I-TNP-albumin + carbohydrate-depleted IgG2b); D, $^{125}$I-labeled control IgG2b; E, $^{125}$I-labeled carbohydrate-depleted IgG2b. Each point represents the mean ± SD from at least four mice expressed as the percentage of the administered dose of radioactivity remaining in circulation 30 min or 90 min after injection. Values for lanes B, C, D, and E were calculated from 50% ammonium sulfate-precipitable radioactivity.

## DISCUSSION

We have attempted to analyze the role of carbohydrate chains on monoclonal antibody molecules in their biological functions. Tm, a select inhibitor of dolichol-dependent glycosylation (9, 28), in combination with an anti-TNP reactive hybridoma cell line, GORK, allowed production and secretion of carbohydrate-deficient IgG2b molecules in useful amounts. Absence of carbohydrate chains in the $C_H2$ domains of IgG2b antibodies (29) induced by this procedure had drastic and select consequences as to antibody effector functions. No impact was noted with regard to fine antigen-binding specificity or ability to react with protein A from *Staphylococcus aureus*. However, carbohydrate removal did reduce, close to completely, some other biological functions of the Ig molecules; namely, ADCC, binding to Fc receptors on macrophages, complement activation, and rapid elimination of antibody–antigen complexes from the circulation.

The $C_H2$ domains of IgG2b are known to be essential for efficient binding to the corresponding Fc receptors on macrophages (14). The binding site for C1q is also localized to the $C_H2$ domain of IgG (27). Yet several sets of results indicate that additional conformational features of Ig molecules contribute to Fc receptor binding and ability to activate complement. For instance, variations in $C_H1$ or the hinge region, or both, may completely eliminate Fc receptor binding capacity (30, 31) and also may affect seriously complement activating capacity (31, 32). On the other hand, the carbohydrate chains in the corresponding human IgG1 molecules not only cover a hydrophobic patch in $C_H2$ domains (33) but extend to involve regions between $C_H1$ and $C_H2$ (34). Thus, it is possible that allosteric changes of IgG molecules because of binding to "rigid" antigens may lead to a carbohydrate chain-dependent change in $C_H2$. The carbohydrate chains in Ig molecules in aggregates or immunocomplexes are also more flexible (35) and accessible (36) than in the native antibody molecule. It is possible that such carbohydrate chains directly participate in the reaction with the Fc receptors or C1q or both. Alternatively, their increased flex-

ibility may merely reflect changes in the polypeptide structure of $C_H2$ domains, making these reactive with Fc receptors or complement factors.

The region between $C_H2$ and $C_H3$ recognized by protein A from staphylococci (23–25) is unchanged in the carbohydrate-lacking antibodies, excluding earlier claims that the Fc receptors and protein A react with the very same steric configurations on IgG molecules (37). Studies on hybrid molecules of IgG2b and IgG2a have indicated that the ability of $C_H2$ domains to exert Fc receptor binding or complement binding, or both, may be indirectly impaired by amino acid changes outside $C_H2$ (30). However, no studies as to the possible role of carbohydrates were performed in these experiments. The present studies and those involving actual modifications of the peptide parts of Ig molecules suggest the possibility of obtaining antibodies devoid of select biological functions. Such reagents not only may have potential therapeutical possibilities but also should help to elucidate basic underlying mechanisms of the biological function of Fc regions of IgG antibodies.

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