Evaluation in Vitro of Adriamycin Immunoconjugates Synthesized Using an Acid-sensitive Hydrazone Linker


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ABSTRACT

A novel method for linking Adriamycin (ADM) to monoclonal antibodies is described in which the 13-keto position of the anthracycline is used as the attachment site to the linker arm. A new ADM acylhydrazone derivative, Adriamycin 13-[3-(2-pyridyldithio)propionyl]hydrazone hydrochloride, which contains a pyridyl-protected disulfide, was synthesized and used for conjugation to monoclonal antibodies (MAbs) that were thiolated with N-succinimidyl 3-(pyridyldithio)propionate or 2-iminothiolane. This resulted in formation of a linker between MAB and drug that contained a disulfide bond. Conjugation conditions were optimized to yield conjugates with high ADM:MAb molar ratios. The final immunonoconjugate yields were found to decrease as the ADM:MAb molar ratio of the conjugates increased. Stability studies indicated that ADM was released from the immunonoconjugates at mildly acidic pHs ranging from 4.5-6.5. Treatment of immunonoconjugates with mild reducing agent dithiothreitol resulted in release of an acylhydrazone derivative of ADM. Flow-cytometric studies showed that the binding activity of various MABs following conjugation to ADM was preserved at ADM:MAb molar ratios up to 10. Antibody-directed cytotoxicity was demonstrated under several assay conditions using combinations of antigen-positive and antigen-negative cells and binding and nonbinding immunonoconjugates. In several experiments, ADM immunonoconjugates were more potent than equivalent amounts of unconjugated ADM.

INTRODUCTION

ADM\(^1\) is an important anti-tumor antibiotic used in the treatment of leukemia, breast carcinoma, and other cancers. However, the efficacy of ADM therapy is limited by the dose-dependent toxic side effects, which include bone marrow suppression and cardiotoxicity (1, 2). Recently, much interest has focused on linking anticancer agents to antibodies directed against tumor-associated antigens in order to improve the therapeutic efficacy of the drugs.

A number of reports have shown that Adriaamycin and the closely related anthracycline Daunomycin can be linked to polyclonal and monoclonal antibodies using many different chemical strategies (for reviews, see Refs. 3–5). The most frequently used approach for attachment has been through the amino sugar moiety of the anthracycline (4, 6). Alternatively, anthracyclines have been directly linked to antibodies through carbodiimide-mediated linkage of amino sugar to carboxyl groups on the antibody (4) and by cross-linking amino groups of the drug and antibody with glutaraldehyde (4, 7). In other studies, the amino sugar of anthracyclines has been linked to high-molecular-weight dextran carriers that were covalently attached to antibodies (8–11).

Modifications of the amino sugar of anthracyclines have been shown to decrease the cytotoxic activity of the drug (12, 13). Drug conjugation strategies utilizing the amino sugar portion of the anthracycline molecule also resulted in a loss of the cytotoxic activity of the conjugated drug (4, 6). Dillman et al. (14) reported that mixtures of doxorubicin and MAB T101 were significantly more efficacious than T101-dextran-doxorubicin conjugates. More recently, an acid-sensitive cis-aconityl linker has been used to attach MABs to the amino sugar of doxorubicin (15) and daunorubicin (16). This conjugation scheme allows for release of unmodified drug under mild acidic conditions from the antibody. Other groups have reported that immunonoconjugates in which anthracyclines were attached to the MAB at the C-14 position retained cytotoxic drug activity, and exhibited selective (antibody-directed) cytotoxicity in vitro (17, 18). Thus, the activity of conjugated ADM appears to be best retained by using linking strategies that preserve the amino sugar portion of the anthracycline.

In this study, we describe conjugation of ADM to MABs using a new methodology that has several advantages over other linker chemistries. ADM-HZN, a new derivative of ADM that contains a pyridyl-protected disulfide attached through the 13-keto position by an acylhydrazone bond, was synthesized. Immunonoconjugates were prepared by reacting ADM-HZN with thiolated MABs resulting in attachment of the drug to the antibody through the 13-keto position of ADM. Synthetic conditions are described that produced immunonoconjugates with drug/antibody molar ratios ranging between 2 and 10. The stability of ADM-HZN immunonoconjugates was studied following treatment with a mild reducing agent and low pH. Immunonoconjugates prepared with a variety of MABs were shown to have retained MAB binding activity to a great extent. Retention of cytotoxic drug activity of conjugated ADM was determined in vitro on human lymphoma and carcinoma cells using colony formation assays. Thus, synthesis of ADM immunonoconjugates by linking MABs to the 13-keto position of ADM represents a new linker strategy in which high cytotoxic drug activity is retained and antibody-directed killing of antigen-bearing tumor target cells can be demonstrated.

MATERIALS AND METHODS

Chemical Reagents. SPDP and 2-IT were obtained from Pierce Chemical Co. Adriamycin-HCl was obtained from Sanraku, Inc. (Japan). All other reagents were of the highest research grade available.

Monoclonal Antibodies. Hybridomas secreting MABs SE9 (anti-transferrin receptor) and T33A1 (anti-M, 40,000 human T-cell antigen) were obtained from the American Type Culture Collection. Antibodies were purified from ascitic fluid produced in BALB/c mice according to the procedure of Bruck et al. (19). Purified MABs G28.5 (anti-M, 50,000 human B-cell antigen), G28.1 (anti-M, 39,000 human B-cell antigen), and L6 (anti-human non-small cell lung carcinoma glycolipid antigen)
were provided by Drs. J. Ledbetter and I. Hellström (Oncogen, Seattle, WA).

Cell Lines. HSB2 (human T-cell leukemia), Daudi (Burkitt lymphoma) and Namalwa (Burkitt’s lymphoma) were obtained from the American Type Culture Collection. HCT116 (human colon carcinoma) was a gift of Dr. M. Brattain. All cell lines were propagated in RPMI 1640 containing 10% fetal bovine serum, penicillin, and streptomycin. Cells were grown at 37°C in a humid atmosphere containing 5% CO2.

Thiolation of MABS. Thiolation of MABS with SPDP was carried out as follows (see Fig. 2). SPDP (50 mM), dissolved in ethanol, was added to MAB (5–10 mg/ml) in PBS to give a final concentration between 10 and 100 mM and the reaction mixture was incubated for 30 min at 30°C. Unreacted SPDP was separated from SPDP-derivatized MAB by gel filtration chromatography using a PD-10 column (Pharmacia). The thiopyridyl protecting groups were removed by reduction with excess DTT. The reduced antibodies were passed through a PD-10 column and the free thiol containing antibodies were used directly before condensation with the Adriamycin hydrazone derivative (described below).

Reactive thiol groups were also introduced onto the MAB protein using 2-IT (see Fig. 2): MABs (5–10 mg/ml in 50 mM triethylamine-50 mM NaCl-1 mM EDTA, pH 8.0) were mixed with 2-IT at a final concentration of 5–10 mM. The reaction was allowed to proceed for 90 min at 4°C. Unreacted MABS separted on a PD-10 column equilibrated with 2 mM NaCl/PBS.

The number of reactive thiol groups incorporated onto the MABS was determined using 5,5′-dithio-bis-(2-nitrobenzoic) acid (ε112 = 14,150), according to the procedure of Ellman (20).

Synthesis of ADM-HZN. To a cooled solution of SPDP (70 mg, 0.22 mmol) in 3 ml of tetrahydrofuran was added 0.3 ml of IM hydrazine solution in isopropyl alcohol. After 20 min of stirring at 0°C, the reaction was followed by a reversed-phase thin-layer chromatography (Adriamycin-HCl (48 mg, 0.083 mmol) were dissolved in 5 ml of methanol and precipitated by addition of acetonitrile to give 45 mg (72%) of 4: m.p. > 125°C darkens its color and not well crystallized. Conjugation reaction was allowed to incubate overnight at 4°C. The reaction mixture was centrifuged at 10,000 × g and the conjugated drug was separated from unreacted ADM by passage through a PD-10 column. The amount of conjugated anthracycline bound to antibody was determined by absorbance at 495 nm (ε495 = 8030). The amount of antibody protein was determined by absorbance at 280 nm (1 mg/ml = 1.4 absorbance units). To correct for the overlap of ADM absorbance at 280 nm, the following formula was used:

\[ \text{MAB (mg/ml)} = \frac{A_{280} - (0.72 \times A_{495})}{1.4} \]
heterobifunctional reagents SPDP or 2-IT and reacting the thiolated MAbs with the ADM-HZN derivative, which was prepared as shown in Fig. 1. The addition of MAbs containing free thiol groups to ADM-HZN, which contained a pyridylthiolated MAb with the ADM-HZN derivative, which was acylhydrazone bond at the 13-keto position of ADM. The synthetic pathway used in the preparation of the immunoconjugates is shown in Fig. 2.

In this scheme, the number of drug molecules covalently bound to the antibody depended on the number of thiol groups introduced on the MAb. Up to 20 thiol groups could be attached to various murine MAbs of the IgG1 and IgG2 subclasses with high protein yields (60–100%). In addition, it was also found that substitution of up to 20 thiol groups onto MAbs did not lead to significant reduction in the binding activity of a number of MAbs (data not shown).

The ADM:MAb molar ratio achieved depended upon the number of thiol groups linked to the MAb and the amount of ADM-HZN derivative added to the thiolated MAb. The scattergram in Fig. 3 shows that ADM:MAb ratios of 3–5 were achieved when ADM-HZN was condensed with MAb containing approximately 7–15 thiol groups. Typically, a 10-fold molar excess of ADM-HZN to protein were added in these reactions. ADM:MAb ratios could be increased by addition of ADM-HZN to MAbs containing 18–25 thiol groups. No significant differences in the ADM:MAb ratios were observed when SPDP or 2-IT was used to thiolate the MAb. However, final protein yields following conjugation of drug to MAb appeared to be somewhat higher with SPDP as compared with 2-IT. Protein yields of 50–80% were commonly obtained for SPDP-thiolated MAbs, whereas yields of 20–50% were more common for 2-IT-thiolated MAbs.

**Binding Activity of ADM Immunoconjugates.** Table 1 shows the retention of MAb binding activity for ADM-HZN immunoconjugates prepared with either the 5E9 or 3A1 MAbs. 5E9 conjugates with molar ratios ranging between 3.5 and 8.5 retained most of their binding activity as compared with unconjugated 5E9. 5E9 immunoconjugates prepared using 2-IT or SPDP had similar binding activities. The 3A1 conjugates prepared using SPDP showed some loss in MAb-binding activity. In general, conjugation of ADM to these and to other MAbs (not shown) resulted in loss of relatively small degrees of MAb-binding activity. We also observed that any significant losses in binding activity and protein yields occurred following reaction of ADM-HZN with thiolated MAb rather than after the introduction of thiol groups onto the MAbs.

**Stability of ADM Immunoconjugate under Acidic Conditions.** Acylhydrazone bonds can undergo hydrolysis under acidic conditions. It should be possible to release unmodified ADM directly from the MAb protein by hydrolysis of the hydrazone bond at the 13-keto position. The stability of an L6 immunoconjugate was determined at various pHs ranging from 4.0 to 7.0 (Fig. 4). After incubation for 24 h, a single product, which had spectral characteristics and column retention time the same as that of the ADM standard, was detected by HPLC. The amount of ADM released from the immunoconjugate after 24 h increased as the pH was lowered from 7.0 to 4.0. Kinetic studies showed that at pH 4.5, the release of ADM from MAb protein is quantitative and rapid (t½ = 2.5 h, data not shown). The immunoconjugates were also found to be quite stable when incubated for 24 h in mouse or human serum (data not shown). It thus appears that this chemical linkage strategy allows for release of unmodified ADM from the ADM-HZN immunoconjugate under mild acidic conditions typically found in endosomal and lysosomal vesicles.

**Reduction of ADM Immunoconjugates with DTT.** In the conjugation procedure, the linker arm is generated by formation of a disulfide bond between the thiolated MAb and ADM-HZN. It should thus be possible to release an anthracycline moiety by treatment of the immunoconjugate with a reducing agent such as DTT. After treatment of an L6 conjugate with 10-fold excess DTT, a single anthracycline product, which had a column retention time different from that of ADM-HCl or ADM-HZN, appeared in the HPLC chromatogram (Fig. 5).

In similar studies, the same product was formed after the reduction of ADM-HZN with dithioerythritol (Fig. 6B). The anthracycline derivative, Adriamycin 13-(3-thiopropionyl)hydrazone, released by reduction upon treatment with acid (pH 2.8) quantitatively yielded unmodified ADM (Fig. 6C). Thus, free ADM can be obtained from ADM immunoconjugates through 2 reaction pathways as depicted in Fig. 7.
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THIOLATING AGENT: SPDP

Fig. 3. A, Scattergram comparing the SH:MAb molar ratio with the final ADM:MAb molar ratio achieved after condensation with ADM-HZN to SPDP-thiolated Mabs. O, Conjugates made using MAb 5E9; •, those made using 3A1. B, Same as A except Mabs were thiolated with 2-IT.

Table 1 Relative binding affinity estimates after ADM conjugation to MAb proteins

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Molar ratio</th>
<th>$[I]_0$</th>
<th>$[I]_5$</th>
<th>$K_{conj}$</th>
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<tbody>
<tr>
<td>SPDP linker</td>
<td>5E9-ADM</td>
<td>3.5</td>
<td>4.2</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>3A1-125I</td>
<td>4.0</td>
<td>6.7</td>
<td>2.1</td>
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<tr>
<td></td>
<td>5E9-ADM</td>
<td>4.9</td>
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<td></td>
<td>3A1-125I</td>
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<td></td>
<td>5E9-ADM</td>
<td>8.5</td>
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<td></td>
<td>3A1-125I</td>
<td>2.6</td>
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<td>5E9-ADM</td>
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<td></td>
<td>5E9-ADM</td>
<td>6.7</td>
<td>7.3</td>
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$[I]_0$, Molar concentration of antibody conjugate giving 50% inhibition of tracer antibody; $[I]_5$, molar concentration of antibody giving 50% inhibition of tracer antibody; $K_{conj}$, relative affinities calculated as

$$K_{conj} = \frac{[I]_5K_{eq}}{[I]_0}$$

where $K_{eq}$ is the equilibrium constant of the unconjugated MAb as determined by Scatchard analysis.

Cytotoxic Activity of ADM Immunoconjugates on Human Lymphoid Tumor Cells. ADM-HZN immunonojugates were tested in vitro for cytotoxicity using colony formation in soft agar. Fig. 8 compares the cytotoxic activity of 5E9-ADM-7.5 and 3A1-ADM-7.0 immunonojugates (thiolated with 2-IT) after 1.5-h incubation with the 5E9 antigen-positive and 3A1 antigen-negative Burkitt's lymphoma cell line, Daudi. Comparison of the dose-response curves shows that the 5E9-ADM-7.5 conjugate, which retained 93% of the original binding activity for antigen-bearing target cells, was significantly more potent than 3A1-ADM-7.0, the nonbinding control conjugate.

The limiting dilution assay, which provides a measure of the log cell kill, was used to test for cytotoxic drug activity of the same two ADM-HZN-immunoconjugates towards Namalwa cells (5E9+, 3A1−) using a longer exposure format (24 h). As described in Fig. 9, 5E9-ADM-7.5 conjugate produced 1–2 logs greater cell kill over a wide concentration range as compared with the nonbinding 3A1-ADM-7.0 conjugate. Up to 5 logs of cell kill were measured at the highest dose. The activity of the 5E9 conjugate was equal to and at several of the lower concentrations greater than an equivalent dose of free ADM. While cytotoxic activity for the nonbinding 3A1 immunoconjugate
was detected, the level of cytotoxicity was less than an equivalent amount of unconjugated ADM.

The 5E9-ADM-7.5 and 3A1-ADM-7.0 conjugates described above were synthesized using 2-iminothiolane as the protein-thiolating agent. Immunospecific cytotoxicity was also observed with immun conjugates that were prepared using SPDP as the thiolating agent. Fig. 10 shows selective cytotoxic activity on Daudi cells of 5E9 and 3A1 immunoconjugates prepared using SPDP as the thiolating agent. Selective cytotoxicity was also to be demonstrated with immunoconjugates prepared with MAbs other than 5E9. As shown in Fig. 11, G28.1-ADM-9.0 conjugate prepared using SPDP was cytotoxic toward 2 antigen-positive cell lines (Daudi, Namalwa) but not the antigen-negative cell line (HSB2). HSB2 cells had comparable sensitivity to ADM (50% inhibitory concentration = 4.5 × 10⁻⁸ M) as Daudi (7 × 10⁻⁸ M).

Cytotoxic Activity: Carcinoma Cells. Preferential killing of antigen-positive cells by 5E9-ADM-7.5 was also observed using an anchorage-dependent carcinoma cell line. As shown in Fig. 12, greater cytotoxicity was observed when the HCT 116 cells (5E9+, 3A1−) were reacted with 5E9-ADM-7.5 as compared with 3A1-ADM-7.0.

DISCUSSION

In this study, we describe a novel method for linking ADM to MAbs via an acylhydrazone bond at the 13-keto position of ADM. Immunoconjugates were constructed by condensation of thiolated MAbs with a new 13-acylhydrazone ADM derivative, ADM-HZN, which contains a reactive pyridyl disulfide bond. This results in formation of a linker arm containing a disulfide bond and more importantly, an acid-sensitive acylhydrazone bond at the 13-keto position of ADM. One of the unique features of this conjugation strategy is that there are 2 cleavable sites in the linker arm that allow for the anthracycline to be released from the MAb protein. Unmodified ADM was shown to be rapidly released by hydrolysis under mild acidic pHs ranging from 4–5.5, which are typically found in endosomal and lysosomal vesicles. The disulfide bond in the middle of the linker arm was shown to be cleaved by mild reducing agents. This leads to release of an ADM derivative, which upon treatment with mild acid generates unmodified ADM. Thus, the high cytotoxic drug activity observed with the ADM immunoconjugates most likely is a direct consequence of the ability of unmodified ADM to be readily released from the MAb protein.

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Fig. 6. Generation of ADM from ADM-HZN following reduction and acid hydrolysis. A. HPLC chromatogram of ADM-HZN; B. HPLC chromatogram following treatment of ADM-HZN with 1.6 m excess diithioerythritol. C. HPLC chromatogram following hydrolysis of the compound in B with pH 2.8 buffer. Arrows, position of ADM-HCI standard eluted under identical chromatographic conditions.

Fig. 7. Chemical pathways for generating unmodified ADM from ADM-immunoconjugates by direct hydrolysis or following reduction of the disulfide bond.
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Fig. 8. Cytotoxicity of ADM (■), 5E9-ADM-7.5 (♦), and 3A1-ADM-7.0 (★) immunoconjugates on the Daudi cell line (phenotype: 5E9+, 3A1−). Immunoconjugates and ADM were incubated with Daudi cells for 1.5 h, washed, and cytotoxicity determined using soft agar colony formation assay as described. Bars, SD of triplicates.

Fig. 9. Cytotoxic effects of 5E9-ADM-7.5 (♦), 3A1-ADM-7.0 (★), and ADM (■) towards Namalwa cells (phenotype 5E9+, 3A1−) as determined by the limiting dilution assay. Cells were incubated with immunoconjugates for 22 h, washed, and log cell kill was determined as described in “Materials and Methods.”

Almost all of the ADM in the conjugate preparations was covalently bound to MAb.

Experimental evidence presented indicated that the cytotoxic activity of the ADM-HZN immunoconjugates could be attributed to delivery of drug to target cells by the MAb. The cytotoxic activity of the ADM immunoconjugates was shown to be greater when tested on antigen-bearing target cells than on non-antigen-bearing cells. Immunospecific cytotoxic effects were observed with conjugates prepared with either SPDP or 2-IT. Two 5E9 in the intracellular environment of tumor cells (described below).

In other reports, immunoconjugates prepared by linking MAb to the amino sugar of anthracyclines through an acid-sensitive cis-aconityl linker were described to have retained high cytolytic activity (15, 16). However, the cis-aconityl linker chemistry can lead to formation of heterogeneous chemical linkages between MAb and drug. One of the advantages of the ADM-HZN chemistry is that uniform chemical linkages between drug and MAb are formed. Ogden et al. (22) reported that 30% of the ADM in the immunoconjugates synthesized using the cis-aconityl linker was noncovalently bound to the MAb. ADM-HZN immunoconjugates have been placed in buffer containing 6 M urea or 1% sodium dodecyl sulfate and free drug was extracted with acetonitrile. Under these denaturing conditions, less than 5% of total drug in the conjugate was found by HPLC in the organic extract (data not shown). These findings, along with the demonstration of quantitative release of drug under acidic and reducing conditions, indicate that...
antigen-positive lymphomas (Daudi, Namalwa) and one 5E9-positive carcinoma (HCT116) were preferentially killed by the 5E9 immunoconjugate but not by the nonbinding control 3A1 immunoconjugate. Immunospecific cytotoxicity was also demonstrated with a G28.1 immunoconjugate, which was cytotoxic toward two antigen-bearing target cells but was not cytotoxic toward the antigen-negative target cells. Binding and cytotoxicity properties of the immunoconjugates reported here appear to represent an improvement over immunoconjugates described in the literature in which ADM or daunomycin were directly linked to antibodies through the amino sugar moiety (3–5).

For drug targeting with MAbs, one method to increase the amount of drug delivered to the tumor cell surface is to attach as many drug molecules to MAb as possible. We were able to optimize the chemistry to yield conjugates that attain ADM:MAb ratios between 6 and 10 using several MAbs of different isotypes. The amount of protein recovered after conjugation of acid-sensitive immunoconjugates contained low ADM:MAb ratios, had impaired antibody binding properties, and exhibited reduced drug activity (4, 6).

Unconjugated ADM-HZN, which has the same 13-acylhydrazone bond as present in ADM-immunoconjugates, was found to be 10 to 50 times less potent than ADM in vitro (data not shown) and in vivo (see accompanying article). However, ADM-HZN conjugates were consistently found to be more potent than free ADM-HZN and in some experiments more potent than parent drug ADM (for example, see Fig. 9). It can be speculated that the high cytolytic activity of ADM immunoconjugates may be due to the mechanism by which antibody-bound drug is delivered to the intracellular environment of the target cell. MAbs 5E9, 3A1, and G28.1 are rapidly internalized following binding to tumor cell surface (data not shown). ADM conjugated to modulating MAbs probably enters the cell through the same endocytic pathway that leads to internalization of membrane-bound antibodies and ligands (for review, see Ref. 24). Internalization of conjugates by this pathway delivers the conjugated ADM-HZN to intracellular acidic endosomal vesicles and lysosomes. The acid-sensitive nature of the acylhydrazone linker in the conjugates would allow for the release of unmodified ADM from the MAb protein in intracellular acidic vesicles. These differences in uptake mechanisms and intracellular trafficking between free and antibody-bound ADM may account for the differences in potency observed in vitro. The mechanism of action of ADM-immunoconjugates and quantitative aspects of ADM delivery are the subject of other studies. Delivery of ADM to cell surface membrane components may also be a contributing factor to the increased potency observed because it has been postulated that the cytotoxicity of ADM is mediated by a membrane effect (25).

In conclusion, we have described a new chemical linker strategy for construction of acid-sensitive ADM immunoconjugates and demonstrated the efficacy and chemical properties of these conjugates in vitro. This methodology for the preparation of acid-sensitive immunoconjugates can be utilized to conjugate more potent 13-keto anthracyclines that contain blocked amino sugar residues [i.e., 3′-deamino-3′-(3-cyano-4-morpholinyl)adriamycin] that cannot be attached using the cis-13-cis isomer of daunomycin with anti-tumor antibodies. Immunol. Rev., 62: 5–27, 1982.

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5. Unpublished data.

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Unpublished data.


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