Monoclonal antibodies against GA_{13}-imide recognize the endogenous plant growth regulator, GA_4, and related gibberellins

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Monoclonal antibody(MAB)-secreting hybridomas were selected from fusions of spleen cells derived from Balb/c mice immunized with the BSA conjugate of GA_{13}-19,20-imide-β-alanine 7-methyl ester with the myeloma line X63.Ag8.653. Selection based on [1,2-^{3}H]GA_4 methyl ester binding capacity yielded two stable hybridoma clones that secreted antibodies of the IgG1 subclass. These MAB exhibited high affinities for GA_4 methyl ester (3.2×10^8 and 1.0×10^9 M^{-1}) which allowed quantitation by HPLC-RIA of nanogram or sub-nanogram amounts of the gibberellins A_2, A_3, A_4, A_7 and A_9 as the methyl esters, in biological fluids.

Gibberellin *Monoclonal antibody* *Plant growth regulator-monoclonal antibody*

1. INTRODUCTION

Gibberellins (GAs) form an important part of the network of chemical signals which regulates nearly every aspect of plant growth and development. However, the abundance of chemically related GAs and exceedingly low levels at which they occur make it extremely difficult to obtain data about their relative distribution in plant tissues and about their levels. MABs have proven useful for the analysis of other plant growth regulators (PGRs; review [1]). Consequently, much interest in the application of this technology to GA analysis has arisen. However, conventional immunogen structures have proven unsuitable to establish hybridomas secreting GA-specific MABs.

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Abbreviations: BSA, bovine serum albumin; GA, gibberellin A; HPLC, high-pressure liquid chromatography; MAB, monoclonal antibody; PGR, plant growth regulator; RIA, radioimmunoassay

Here, we report a novel approach which allows production of GA-specific MABs of high affinity that are useful for GA immunoassay, immunoaffinity chromatography and the generation of anti-idiotypic antibodies.

2. MATERIALS AND METHODS

Female 6–8-week old, Balb/c mice were immunized with BSA-conjugated GA_{13}-19,20-imide-β-alanine 7-methyl ester (50 μg per injection; 2 mol GA per mol protein; I. Yamaguchi et al., in preparation) using the schedule described in [2]. Similar immunizations with GA_1- and GA_3-(C-7)-BSA and GA_3-3-succinoyl-BSA (200 μg per injection) were also carried out. Serum titers were determined by RIA [3]. The following tracers were used as the methyl esters throughout this study: [1,2-^{3}H]GA_1 (NEN, 1.4×10^{12} Bq·mmol^{-1}, or obtained from Dr R. Atzorn, Glasgow, 3.7×10^{11} Bq·mmol^{-1}), [1,2-^{3}H]GA_4 (Amersham, 1.4×10^{11} Bq·mmol^{-1}) and [17-nor,16-ol-^{3}H]GA_3 (2.2

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$\times 10^{11}$ Bq·mmol^{-1} [3]). Four days prior to fusion, a final booster immunization was administered.

Fusions were performed with spleen cells of immunized mice and cells of the myeloma line X63.Ag8.653 (ratio 4:1) as described [2,4,5]. Cell growth was usually observed 14 days after fusion. The presence of GA-specific antibodies was monitored by RIA using [1,2-^{3}H]GA_4 methyl ester as tracer when checking for antibodies raised against the GA_{13}-hapten. Positive cell populations were purified by re-cloning in limiting dilution (3 ×). The hybridoma clones were checked for stability and ability to induce ascites fluid in Balb/c mice. Immunoglobulin subclass was determined by Ouchterlony double-immunodiffusion [5].

HPLC analysis was performed [6] with minor modifications in the developing solvent. Methanolic samples (80 μl) were injected into a Nucleosil-N$(CH_3)_2$ column (150 × 4 mm i.d.) and eluted isocratically with methanol (99%), water (0.95%) and acetic acid (0.05%) at a flow rate of 1.0 ml·min^{-1} and a pre-column pressure of 100 bar. Fractions of 1.0 ml were collected, dried, treated with ethereal diazomethane [7] and redissolved in 1 ml phosphate-buffered saline, pH 7.4. Aliquots were then taken for isotope recovery analysis (39.2% for GA_4 and 28.9% for GA_1) and RIA [3].

Sphaceloma manihoticola (ATCC 44292), grown on malt extract agar, was kindly provided by Dr W. Rademacher, BASF Ludwigshafen, FRG. Agar slants (28 g) were extracted, after the addition of [1,2-^{3}H]GA_1 (7.0 pmol) and [1,2-^{3}H]GA_4 (0.8 pmol) as internal standards, with 30 ml methanol at 4°C (1 × 16 h, 2 × 1 h). The extracts were further purified by passage, in 70% methanol, through a SepPak C_{18} cartridge (Waters), followed by removal of the organic solvent, acidification to pH 2.5 and extraction of the GAs with ethyl acetate. After drying, the samples were redissolved in methanol and subjected to HPLC.

3. RESULTS

3.1. *Hybridoma production*

In contrast to the situation in rabbits, none of the animals immunized with either GA_1 or GA_3 linked to BSA via C-7 produced detectable antibody titers. Likewise, GA_3-succinoyl-BSA did not elicit an immune response. On the other hand, serum from all animals immunized with the BSA conjugate of GA_{13}-19,20-imide-β-alanine 7-methyl ester showed significant binding of [1,2-^{3}H]GA_4 methyl ester after 2 weeks of immunization. No binding of [1,2-^{3}H]GA_4 and only slight binding of [1,2-^{3}H]GA_1 methyl ester was observed. Out of a series of 10 fusions, one yielded 3 positive wells (1.6% of all wells) from which stable hybridomas (J51-I-B1, J51-II-C1 and J51-III-A4) were obtained. J51-I-B1 was not characterized further because of its low affinity for GA_4 methyl ester. Both J51-II-C1 and J51-III-A4 secrete MAB of the IgG1 sub-type. From a saturation analysis with

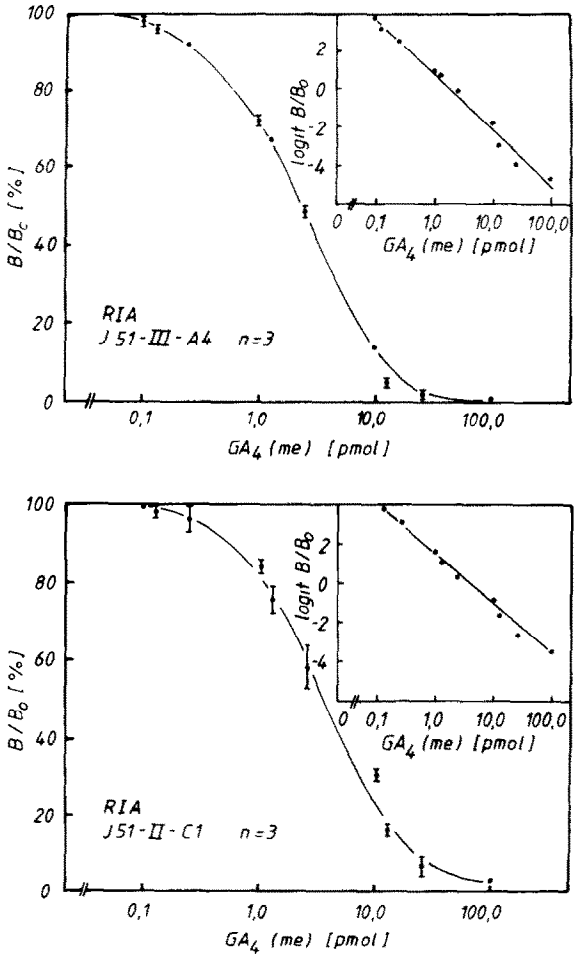

Fig.1. Representative radioimmunoassay standard curves (for n = 3 standard replicates) of MAB J51-II-C1 and J51-III-A4. GA_4(me), GA_4 methyl ester.

[1,2-^{3}H]GA_4 methyl ester, Scatchard plots gave affinity constants of 1.0×10^9 M^{-1} (J51-II-C1) and 3.2×10^8 M^{-1} (J51-III-A4).

3.2. *Immunoassay*

The RIA technique previously devised for GA antisera [3] was successfully adopted for the present study. Ascites fluid (1:45000 final dilution) from both hybridomas bound 30% of an added 83 fmol (117 Bq) of [1,2-^{3}H]GA_4 methyl ester under standard conditions. Typical standard curves for both MAB are shown in fig.1. Measuring ranges extend from 0.5 to 10 pmol for GA_4 methyl ester and coefficients of variation for triplicate standards were in the range 1–5%.

For cross-reaction analysis, a range of GAs with various substitution patterns in either the A-ring or the C/D-rings was selected. The values given in table 1 were derived from tracer displacement curves at 50% displacement using [1,2-^{3}H]GA_4 methyl ester and increasing amounts of the respective standards, and are expressed on a molar basis. The data reveal a clear-cut pattern of molecular features required for immunoreactivity (fig.2), the highest affinities being observed for 1,2-unsaturated, 3β-hydroxylated, 13-non-hydroxylated C19-GAs with a γ-lactone ring. The presence of a

Table 1

Cross-reactions of gibberellin-directed monoclonal antibodies

Gibberellin	Cross-reaction (%)	
	MAB J51-II-C1	MAB J51-III-A4
A_1	1.0	0.7
A_2	46.3	42.0
A_3	37.0	76.4
A_4	100	100
A_7	805	764
A_9	26.4	52.5
A_{13}	6.7	14.0
A_{14}	<0.3	<0.3
A_{20}	1.5	1.8
A_{34}	14.8	26.3
A_{37}	360	35.6
A_{51}	3.5	6.2
A_{53}	3.2	4.2

The data are cross-reactions in percent on a molar basis. All compounds were assayed as the methyl esters

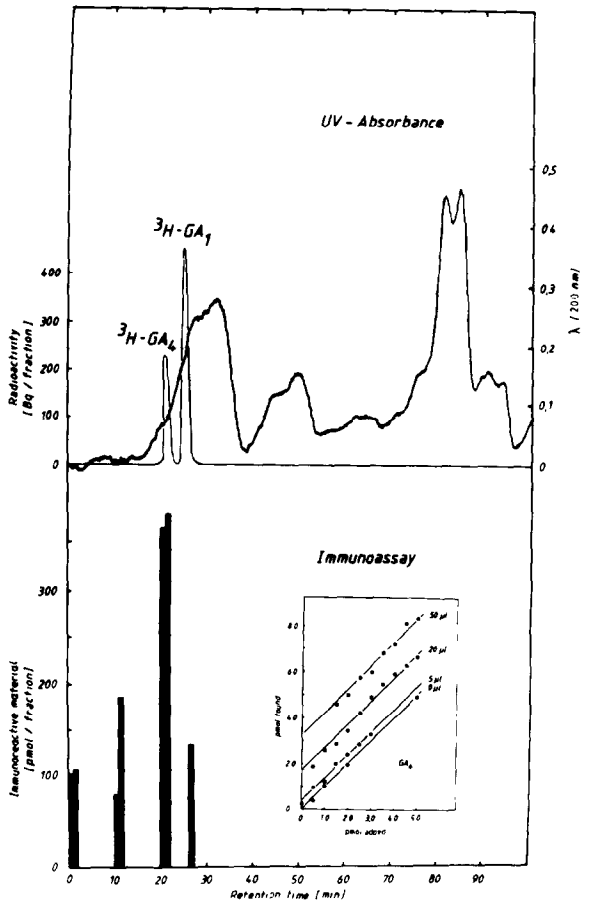

Fig.2. Immunohistogram of a culture diffusate of *Sphaceloma manihoticola* after fractionation by HPLC on Nucleosil-N$(CH_3)_2$. Immunoreactivity expressed as pmol per fraction of GA_4 methyl ester equivalents. (Inset) Determination of analytical recovery of GA_4 in *S. manihoticola* culture diffusates. The crude diffusate, worked up as described but not fractionated by HPLC was, after methylation, assayed in various aliquot volumes in the presence of unlabeled standard GA_4 methyl ester. Slopes of regression lines: standard alone, 1.01; 5 μl, 1.02; 20 μl, 1.12; 50 μl, 1.13; test for parallelism of dilution curve and standard curve from plot of aliquot size (x-axis) vs GA_4 found in the absence of standard (y-axis): $r = 0.999$.

13β-OH group strongly reduces affinity. A similar tendency is observed in the polyclonal antisera raised in rabbits against the same immunogen (I. Yamaguchi et al., in preparation).

The applicability of the MABs for GA analysis was demonstrated by an HPLC-RIA analysis of

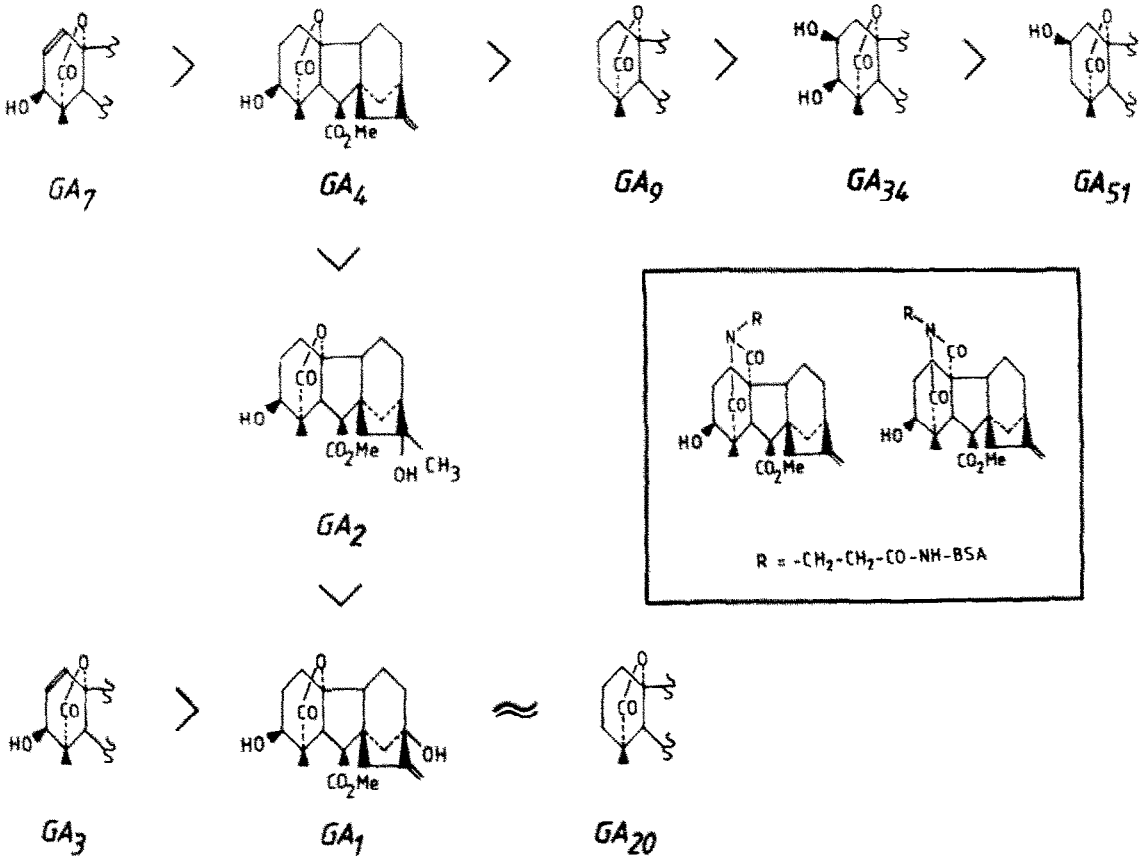

Fig.3. Structural requirements for immunoreactivity of δ-lactone gibberellins with MAB J51-II-C1 and J51-III-A4. (Inset) Structure of the GA_{13}-19,20-imide-β-alanine 7-methyl ester BSA conjugate.

immunoreactivity in marginally pre-purified extracts of *S. manihoticola*, a phytopathogenic fungus causing the superelongation disease of cassava [8]. The fungus was shown to produce predominantly GA_4, small quantities of GA_{13}, GA_{14}, GA_{29} and GA_9 and traces of GA_{15}, GA_{25}, GA_{36} and GA_{37}. In contrast to *Gibberella fujikuroi*, GA_1, GA_3 and GA_7 were not found in the culture medium [9]. Fig.2 shows 4 distinct areas of immunoreactivity demonstrating that, as expected, this MAB (J51-II-C1) is not monospecific. On the other hand, the HPLC system used here separates GA_4 completely from the other cross-reactants present in this material. Although the sample is still rather crude (as evident from the UV trace), the immunological signal of GA_4 is clear-cut while the UV signal is fully concealed. Dilution analysis of an unseparated, methylated *Sphaceloma* extract in the presence of standard amounts of GA_4 methyl ester (fig.2, inset) gave quantitative recovery indicating the absence of sample interference in the process of antigen-antibody binding.

4. DISCUSSION

This report demonstrates the feasibility of raising MABs against selected groups of GAs. In order to cope with the abundance of GA structures in plants, group-selective antibodies in conjunction with high-performance separation techniques appear more appropriate for immunological GA analysis than a large set of monospecific antibodies. Experience with GA antisera has, in addition, shown that the structural similarities of certain GAs make the generation of monospecific antibodies difficult [3] (and S. Kurogochi et al., in

preparation). Thus, in re-designing GA-hapten structures for the murine immune system, a 'group-selective' approach was favoured. Since the substituents responsible for physiological activity of the GA molecule, as well as for its inactivation, are exposed to the β-plane of the GA structure and since biosynthetic pathways are mainly discernible due to the sequence of introduction of β-hydroxls (review [10]), a conjugate structure exposing the GA β-plane while at the same time leaving it structurally unchanged was considered optimal. GA_{13}-19,20-imide-β-alanine 7-methyl ester ideally fulfilled these requirements. It mimics the structure of, e.g. GA_4 methyl ester, and, after coupling to a protein, leaves the β-plane of the GA exposed while directing the α-plane to the protein surface, even though two isomeric forms are possible (fig.3, inset). The conjugate also proved to be highly immunogenic in Balb/c mice. As expected, MABs directed against this hapten recognize GA_4 methyl ester, but are only slightly reactive with C-13 hydroxylated GAs such as GA_1 methyl ester. Thus, a clear-cut group-selective affinity of both MABs characterized in this study is apparent (fig.3). The structural requirements for optimum antibody binding of C19-GAs with a γ-lactone are (i) a β-OH in the 3-position, (ii) a 1,2-double bond and (iii) the absence of the 13-OH group. The absence of a 3β-OH still renders the compound immunoreactive if the other requirements for activity are fulfilled (e.g. GA_9) while the presence of a 2β-OH interferes with antibody binding. In the case of GA_3, the effect of the 13-OH is apparently counteracted by the strong effect of the 1,2-double bond. Thus, in contrast to GA_1, GA_3 is significantly cross-reactive. The influence of the 1,2-double bond on affinity is not completely understood since GA_{13} lacks this function. A possible explanation could be that in the hapten, the 1- and 2-positions are bordered, although from the α-plane only, by π-electrons from the C-19 carboxyl oxygen and from the imino nitrogen. As expected, δ-lactone GAs such as GA_{37} are also highly cross-reactive. This strengthens the conclusion that binding to these MABs is primarily directed by the β-substituents of a GA. A lactone ring, however, is required for full immunological activity, since GA_{13} and GA_{14} are both only weakly reactive. This finding is compatible with the proposed structure of the immunogen (fig.3, inset).

In summary, the MABs described here clearly identify a narrow group of systematically related GAs and will be useful for the immunoassay of these GAs. Furthermore, purification of GAs by immunoaffinity chromatography as well as the generation of anti-idiotypic antibodies now seem feasible.

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REFERENCES

[1] Weiler, E.W. (1986) in: Modern Methods of Plant Analysis (Linskens, H.F. and Jackson, J.F. eds) vol.5, Springer, Berlin, in press.
[2] Eberle, J., Arnscheidt, A., Klix, D. and Weiler, E.W. (1986) Plant Physiol., in press.
[3] Atzorn, R. and Weiler, E.W. (1983) Planta 159, 1–6.
[4] Galfre, G. and Milstein, C. (1981) Methods Enzymol. 73, 1–46.
[5] Mertens, R., Deus-Neumann, B. and Weiler, E.W. (1983) FEBS Lett. 160, 269–272.
[6] Yamaguchi, I., Fujisawa, S. and Takahashi, N. (1982) Phytochemistry 21, 2049–2055.
[7] Weiler, E.W. and Wieczorek, U. (1981) Planta 152, 159–167.
[8] Rademacher, W. and Graebe, I.E. (1979) Biochem. Biophys. Res. Commun. 91, 35–40.
[9] Rademacher, W., Jung, J., Laatsch, H., Rave-Fränk, M., Hedden, P. and Graebe, I.E. (1982) 11th Conf. Int. Plant Growth Subst. Assoc., Aberystwyth, Wales.
[10] Sponsel, V. (1983) in: The Biochemistry and Physiology of Gibberellins (Crozier, A. ed.) pp.151–259, Praeger, New York.