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CHARACTERIZATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR ISOPENTENYL ADENOSINE DERIVATIVES OCCURRING IN TRANSFER RNA

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SUMMARY: A family of isopentenyl adenosine derivatives are naturally occurring components of transfer RNA and are involved in several different functional roles in the cell. To facilitate the study of the biochemistry of these modified nucleosides we have raised monoclonal antibodies to N^6-(Δ^2-isopentenyl)adenosine and N^6-(4-hydroxy-3-methyl-but-2-enyl)adenosine. The antibodies show considerable specificity and three characteristic types are distinguishable. The first type have the hydroxylated derivative as the preferred antigen, the second type have isopentenyl adenosine as the preferred antigen and a third type show a specificity for all isopentenyl-containing derivatives.

N^6-(Δ^2-isopentenyl)adenosine (i^6A) and its derivatives are components of transfer RNA from all phylogenetic classes of organisms (1). Members of this family of modified nucleosides (See Fig. 1) are located adjacent to the 3' end of the anticodon and occur only in transfer RNA molecules which respond to codons of the type UXX (2). Several diverse functional roles have emerged for these hydrophobic adenosine derivatives including the interaction between transfer RNA and ribosomes (3), the regulation of transcription by attenuation (4), the uptake of aromatic amino acids (5) and the adaptive response by enteric bacteria to growth in an iron-restricted environment (6). In addition, i^6A itself is a potent plant hormone (cytokinin) and possesses some anti-tumor activity (7).

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Abbreviations: i^6A, N^6-(Δ^2-isopentenyl)adenosine; pi^6A, i^6A 5'-monophosphate; ms^2i^6A, N-^{6}(Δ^2-isopentenyl)-2-methylthioadenosine; io^6A, N^6-(4-hydroxy-3-methyl-but-2-enyl)adenosine or zeatin riboside; ms^2io^6A, 2-methylthiozeatin riboside; m^6A, N^6-methyl adenosine; m^1A,N^1-methyladenosine; m_2^6A,N^6-dimethyladenosine; m^5U, 5-methyluridine; PVC, polyvinylchloride.

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^{14}C-dihomo-γ-linolenic acid, specific activity 40-60 mCi/mmol (New England Nuclear, Boston, MA). Following ether extraction and separation by silicic acid chromatography, the ether/methanol 95/5 v/v eluate was evaporated to dryness, dissolved in methanol and treated with diazomethane as previously reported (1). Samples were injected into a Waters high performance liquid chromatography (HPLC) equipped with a model U6K injector, a model M-45 solvent delivery system, a model 450 variable wavelength detector and an Omni Scribe B-500 recorder (Houston Instruments, Austin, TX). Compounds were eluted from a straight phase preparatory column (30 cm x 7.8 mm ID, packed with Porasil), using hexane/isopropanol 96/4 v/v as the solvent system at 4 ml/min. Peaks absorbing at 270 nm were collected and the solvent was evaporated in vacuo. The residue was dissolved in 1 ml of methanol and subjected to UV spectroscopy and gas chromatography - mass spectrometry (GC-MS). A small portion (5%) of the samples was injected into a straight phase analytical column (30 cm x 3.9 mm ID, packed with Porasil) and eluted at 1 ml/min with the same system. Other samples obtained from similar experiments were injected as free acids into a reversed phase Radial-PAK cartridge (C_{18}, 8mm ID x 10 cm, 5 µ) in a Z-module system and eluted with methanol/water/acetic acid 70/30/0.01 v/v/v at 4 ml/min. The UV detector was set at 270 nm for the first 10 minutes and at 235 nm for 20 additional minutes. Fractions were collected every minute for counting of radioactivity. All radioactive peaks were subjected to UV spectroscopy and GC-MS.

The effect of 10^{-5} M indomethacin (Sigma, St. Louis, MO) and 5 µM ionophore A23187 (Calbiochem-Behring Corp., La Jolla, CA) on the products of dihomo-γ-linolenic acid by human PMNL was investigated with incubations at 37°C for 15 minutes (indomethacin) or 5 minutes (ionophore). Samples were injected into the Z-module system as described above.

Procedures for UV spectroscopy and GS-MS (6), including catalytic hydrogenation (1) have been previously reported.

RESULTS

A straight phase HPLC chromatogram of a methylated extract obtained by incubating human PMNL with dihomo-γ-linolenic acid is shown in Figure 1. Six peaks, absorbing at 270 nm can be appreciated. All compounds exhibited a typical UV leukotriene spectrum with absorption bands at 258, 268 and 278 for peaks I, IV, V and VI, at 262, 272 and 282 for peak II and at 260, 270 and 280 for peak III. The equivalent chain lengths (C values) were as follows: I = 23.8, II = 24.2, III = 24.9, IV = 24.9, V = 24.2 and VI = 24.9. The mass spectra of compounds I, IV, V and VI were the same. Analysis of the mass spectra of the methyl ester trimethylsilyl ether derivatives of the unsaturated compounds (Figure 2) and saturated compounds (Figure 3) allowed the identifiation of I, IV, V and VI as isomers of 8,15-dihydroxy-9,11,13-icosatrienoic acid. Informative ions for the unsaturated derivative (Figure 2) were: 481 (M-15; loss of CH_3), 465

Preparation of conjugates and monoclonal antibodies.

The nucleosides were coupled to mouse serum albumin (MSA) or bovine serum albumin (BSA) using the procedure of (10). Only those conjugates containing more than 10 moles of nucleoside per mole of serum albumin were used in the studies described below. Two mice were given 3 injections of 25 µg of either an i^6A or trans-io^6A BSA conjugate over a period of 3 months. The first injection (intraperitoneally) was an emulsion in complete Freund's adjuvant; the second (intraperitoneally) in incomplete Freund's adjuvant and the final injection was given intravenously. Three days later the spleens were removed and the splenocytes were cultured for 24 hours in 20 µg/ml of lipopolysaccharide. This procedure resulted in a 3 to 10 fold increase in the total number of clones produced (unpublished observations). The fusion protocol and culture conditions have been described previously except that all media contained 0.1 µM sodium selenite (11). Testing was initiated after 10 days using a Solid Phase Radio-Immune Assay (SPRIA - see below). From each spleen approximately 2,000 clones developed of which a total of 75 were positive for either i^6A or io^6A. Of these only 19 (25%) did not cross-react with the other antigen. Twenty-three hybridomas were chosen for further study and 7 of these were successfully cloned by limiting dilution and stored frozen in liquid nitrogen.

Solid Phase Radio-Immune Assay: This was performed as described previously (11) except that the wells (polyvinyl chloride) were coated with 2 µg/ml of the appropriate nucleoside - MSA conjugate and the I^{125} labelled goat anti-mouse second antibody was purchased from New England Nuclear. In general, the results which are the average of quadruplicate determinations were reproducible to within ± 20% and a ten-fold dilution of a hybridoma supernatant gave 4,000 - 6,000 c.p.m. when tested against the preferred antigen. For the competition experiments the competing nucleoside or nucleotide at various concentrations was added together with the hybridoma supernatant before continuing the assay as usual.

RESULTS AND DISCUSSION

Table 1 details the results of the SPRIA assays for the hybridomas binding to various nucleoside-MSA conjugates. Since the ratio of moles nucleoside/

TABLE 1. Specificity pattern of monoclonal antibody binding to various nucleoside conjugates as measured by SPRIA.

Clone	Nucleoside conjugate[a]					
	i^6a	ms^2i^6A	trans-io^6A	trans-ms^2io^6A	cis-ms^2io^6A	A
JEL 75[b]	7	<5	100	6	6	7
JEL 80	6	<5	100	<5	74	29
JEL 73	100	<5	6	<5	<5	<5
JEL 74	100	<5	9	<5	<5	<5
JEL 77	100	<5	<5	<5	<5	<5
JEL 76	98	85	71	100	59	<5
JEL 79	90	87	83	95	100	5

[a] Nucleosides were conjugated to mouse serum albumin as described in ref. 10.

[b] All of the clones listed showed less than 5% binding when tested against conjugates made with m^6A, m^1A, m_2^6A, m^5U, guanosine, inosine, and cytidine.

moles MSA varies between conjugates and also since different conjugates may stick to the PVC wells to different extents, this data only reveals different patterns of binding. However, it is clear that the antibodies show considerable specificity and three characteristic types are distinguishable. The first exemplified by JEL 75 and 80 have trans-io^6A as the preferred antigen and binding to i^6A is only just detectable. However, JEL 80 recognizes cis-ms^2io^6A whereas JEL 75 does not. The second family consisting of JEL 73, 74 and 77 have i^6A as the preferred antigen but the binding to io^6A is weak. Furthermore, neither ms^2i^6A nor ms^2io^6A(cis or trans) are recognized by this group. Finally, in the third class, JEL 79 and JEL 76, the antibodies bind well to all isopentenyl-containing derivatives although their patterns of specificity are slightly different. That is, JEL 79 binds best to cis-ms^2io^6A whereas for JEL 76 the preferred antigen is the trans isomer. In all cases, there was no detectable binding to conjugates of guanosine, cytidine, inosine, m^1A, m^6A or m_2^6A but surprisingly, JEL 75, 80 and 79 showed a weak interaction with adenosine itself.

In order to study the specificities in more detail we performed competition binding studies (Fig. 2). In particular, we wished to assess the degree of cross-reactivity between i^6A and io^6A as well as to investigate the apparent weak binding of JEL 75, 80 and 79 to adenosine itself. This latter aspect is very important since there is far more adenosine in tRNA than io^6A. Fig. 2a shows that the binding of JEL 75 to the trans-io^6A conjugate is 50% inhibited by 500 pm of trans-io^6A whereas approximately 12.5 nm of i^6A are required to reach the same level of inhibition. This suggests that the binding constant of JEL 75 to trans-io^6A is approximately 25-fold greater than to i^6A. The difference may correspond to the formation of a hydrogen bond in the antibody trans-io^6A complex which cannot form in the case of i^6A. Surprisingly, the i^6A nucleotide competes more weakly than i^6A itself and the binding of adenosine cannot be detected in these competition experiments. Similarly, no binding of adenosine to JEL 79 and 80 could be detected by competition (data not shown). Thus, the weak binding of JEL 75, 79 and 80 to

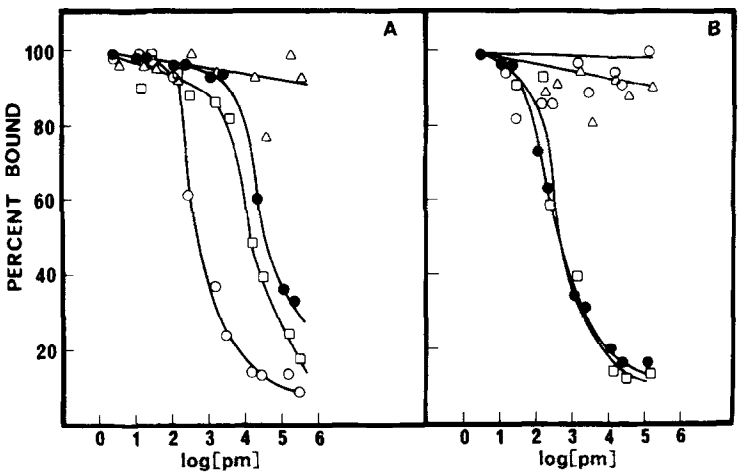

Fig. 2. Competition experiments showing the binding of monoclonal antibody to various modified adenosine conjugates. (a) clone JEL 75. The PVC plates were coated with the trans-io^{6}A MSA conjugate. (b) clone JEL 77. The PVC plates were coated with the i^{6}A MSA conjugate. The competitors were as follows: o—o, trans-io^{6}A; □--□, i^{6}A; ●--●, pi^{6}A; Δ--Δ, adenosine.

adenosine shown by the SPRIA assay must be due to some as yet undetermined property of the adenosine-MSA conjugate.

Competition experiments for JEL 77 are shown in Fig. 2b. In this case there is no significant difference between i^{6}A and the i^{6}A nucleotide and no inhibition can be detected with trans-io^{6}A. Thus, the absolute specificity shown by this antibody for i^{6}A presumably means the hydroxyl group of trans-io^{6}A is excluded from the binding site of the antibody.

Finally, we were also interested to discover if the antibodies which bound to trans-io^{6}A would also recognize cis-io^{6}A. This is shown in Figs. 3a and b where it can be seen that for JEL 80 the trans derivative competes nearly an order of magnitude more effectively than cis-io^{6}A. Comparison of Fig. 3a with Fig. 2a shows that JEL 75 also has a similar prefrence for the trans isomer. From Fig. 3b it is clear that JEL 79 and JEL 76 also bind cis-io^{6}A. Thus, they bind to all isopentenyl adenosine derivatives regardless of the type of modification. Their broad pattern of specificity (see Table 1) is rather curious and it is difficult to visualize what chemical features of i^{6}A and its derivatives are being recognized.

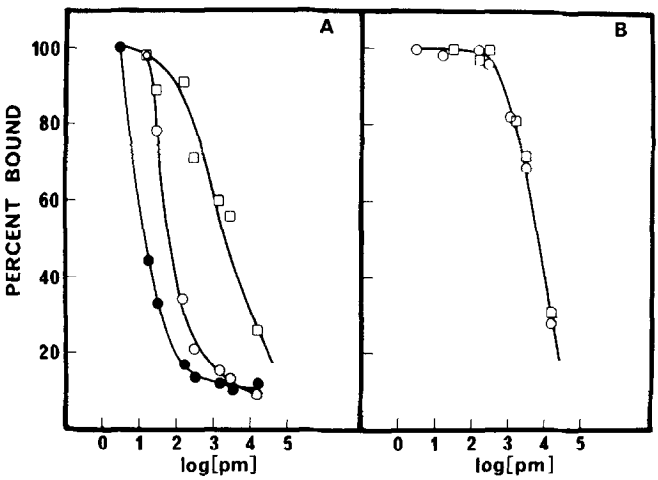

Fig. 3. Competition experiments showing the binding of monoclonal antibody to cis and trans-io^{6}A. The PVC plates were coated with the trans-io^{6}A MSA conjugate. (a) ●—● clone JEL 80, competition with trans io^{6}a; o--o clone JEL 80, competition with cis-io^{6}A; □—□ clone JEL 75 competition with cis-io^{6}A: (b) o--o, clone JEL 79 and □--□ clone JEL 76 competition with cis-io^{6}A.

CONCLUSION

Several monoclonal antibodies have been prepared which show considerable specificity for either i^{6}A or io^{6}A. Two of these antibodies can distinguish between the trans and cis isomers of io^{6}A. In addition, two monoclonal antibodies were prepared which bind to all members of the isopentenyl adenosine family. These monoclonal antibodies will be extremely useful in elucidating the roles of isopentenyl adenosine derivatives in tRNA.

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REFERENCES

1. Hall, R.H. (1970) Prog. Nuc. Acid Res. Mol. Biol. 10, 57-86.
2. Sprinzl, M., and Gauss, D.H. (1982) Nuc. Acids Res. 10, r1-r55.
3. Gefter, M.L., and Russell, R.L. (1969) J. Mol. Biol. 39, 145-157.
4. Buck, M., and Griffiths, E. (1982) Nuc. Acids Res. 10, 2609-2624.
5. Buck, M., and Griffiths, E. (1981) Nuc. Acids Res. 9, 401-414.
6. McLennan, B.D., Buck, M., Humphrey, J., and Griffiths, E. (1981) Nuc. Acids Res. 9, 2629-2640.
7. Skoog, F., and Armstrong, D.J. (1970) Ann. Rev. Plant Physiol. 21, 359-384.
8. Milstone, D.S., Vold, B.S., Glitz, D.G., and Shutt, N. (1978) Nuc. Acids Res. 5, 3439-3455.
9. Vold, B.S., and Nolen III, H.W. (1979) Nuc. Acids Res. 7, 971-980.
10. Erlanger, B.F., and Beiser, S.M. (1964) Proc. Natl. Acad. Sci. USA 52, 68-74.
11. Lee, J.S., Lewis, J.R., Morgan, A.R., Mosmann, T.R., and Singh, B. (1981) Nuc. Acids Res. 9, 1707-1721.