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New Triterpenes, Myrrhanol A and Myrrhanone A, from Guggul-Gum Resins, and their Potent Anti-Inflammatory Effect on Adjuvant-Induced Air-Pouch Granuloma of Mice

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Abstract—Myrrhanol A, a new triterpene isolated from guggul (*Balsamodendron* or *Commiphora mukul* Hook.)-gum resin, displays a potent anti-inflammatory effect on exudative pouch fluid, angiogenesis, and granuloma weights in adjuvant-induced air-pouch granuloma of mice. Its effects were more marked than those of hydrocortisone and the 50% aqueous methanolic extract of the crude drug. Myrrhanol A is a plausible candidate for a potent anti-inflammatory agent. © 2001 Elsevier Science Ltd. All rights reserved.

Guggul-gum resins are prescribed in the form of direct mixtures with powders or extracts of other crude drugs for use as anti-obesity, anti-inflammatory, antibacterial, anticoagulant, and anti-atherosclerosis agents as Ayurvedic folk medicines in India. The crude drugs, guggul and myrrh, are natural resins of gum secreted by *Balsamodendron* (or *Commiphora*) *mukul* Hook. and *Balsamodendron* (or *Commiphora*) *myrrha* Nees, respectively, of the Burseraceae family. Guggul is produced by drying the milky-white sap of the tree (15–20 years old) for one year. Guggulsterone, a compound isolated from guggul, activates lipolytic enzymes, inhibits hepatic cholesterol biosynthesis, and reduces the total serum lipid and total serum cholesterol levels.[1,2] In antiquity, myrrh was used by the Egyptians for embalming and by the Jews as anointing oil. The sesquiterpene constituents isolated from myrrh, furanoeudesma-1,3-diene, and curzarene have analgesic effects that are blocked by naloxone, explaining the use of myrrh as a painkiller in ancient times.[3] However, because of its toxicity, myrrh is not used in medicines today, except as a mouth wash in India.

In the present study, the 50% aqueous methanolic extract was found to exhibit an anti-inflammatory effect on adjuvant-induced air-pouch granuloma in mice. Five new triterpene constituents, myrrhanols A (**1**), B, and C, and myrrhanones A (**2**) and B, together with three known constituents were isolated from the 50% aqueous methanol extract. This paper deals with the isolation and characterization of myrrhanol A (**1**) and myrrhanone A (**2**) and their anti-inflammatory effect. In addition, the anti-inflammatory effects of the guggul extracts were compared with those of myrrh.

Results and Discussion

Isolation and elucidation of the structure of myrrhanol A (1) and myrrhanone A (2)

The gum resins of guggul (*Balsamodendron mukul* Hook.) (Rajastan, India) and myrrh (*Balsamodendron*

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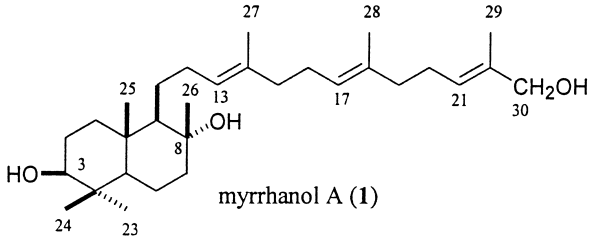
myrrha Nees) (Afghanistan) were purchased from Sharangdhar Pharmaceuticals PVT. Ltd (Pune, India) and were extracted by refluxing with 100% methanol or 50% aqueous methanol. The 50% aqueous methanolic extract of the resin of *B. mukul* was partitioned in an ethyl acetate–water mixture. The ethyl acetate-soluble portion was subjected to normal-phase and reversed-phase silica gel column chromatography and finally to HPLC to give myrrhanol A (**1**, 0.0084%) and myrrhanone A (**2**, 0.026%) (Chart 1).

Myrrhanol A (**1**), $[\alpha]_D^{27}$ +12.2° (MeOH), $C_{30}H_{52}O_3$,[4] positive-ion FAB-MS: *m/z* 483 $(M+Na)^+$, yielded absorption bands in its IR spectrum due to the hydroxyl functions at 3432 and 1046 cm^{-1}. The proton and carbon signals in the ^1H NMR ($CDCl_3$) and ^{13}C NMR (Table 1) spectra[5] of **1** revealed the presence of a methine [δ 3.21 (dd, $J=4.9$, 11.6 Hz, 3-H)] and a methylene [δ 3.96 (s, 30-H_2)] bearing an oxygen function, and three tri-substituted olefins [δ 5.12 (dd, $J=5.8$, 6.7 Hz, 17-H), 5.16 (dd-like, 13-H), 5.38 (dd-like, 21-H)] together with a quaternary carbon bearing an oxygen function [δ_C 73.9] and seven tertiary methyls. The position of the above mentioned functional groups was clarified by the HMBC experiment, which showed long-range correlations between the following protons and carbons: 23, 24-H_3 and 3, 4, 5-C; 25-H_3 and 1, 5, 9, 10-C; 26-H_3 and 7, 8, 9-C; 27-H_3 and 13, 14, 15-C; 28-H_3 and 17, 18, 19-C; and 29-H_3 and 21, 22, 30-C. The phase-sensitive NOESY experiment on **1** showed NOE correlations between the following protons: 23-H_3 and 3, 9-H; 25-H_3 and 24, 26-H_3 (Fig. 1). The stereostructure of myrrhanol A (**1**) was determined on the basis of this evidence. In order to clarify the absolute stereostructure of **1**, **1** was subjected to a modified Mosher's method.[6] The signals due to the protons attached to C-1, 2, and 25 in the ^1H NMR spectrum of the 3-(*S*)-MTPA ester (**1b**) were observed at higher fields than those of the 3-(*R*)-MTPA ester (**1a**) (Δδ: negative), while the signals due to the proton on C-23 and 24 of the 3-(*S*)-MTPA (**1b**) were observed at lower fields than those of the 3-(*R*)-MTPA (**1a**) (Δδ: positive), as shown in Figure 2. Based on the above evidence, the absolute stereostructure of myrrhanol A (**1**) was determined to be (3*S*,5*S*,8*R*,9*R*,10*S*)-3,8,30-trihydroxypolypoda-13*E*,17*E*,21*E*-triene.

The carbon signals in the ^{13}C NMR (Table 1) spectrum[5] of myrrhanone A (**2**) ($[\alpha]_D^{28}$ +11.9° (MeOH), $C_{30}H_{50}O_3$,[4] IR (KBr): 3453, 1650, and 1080 cm^{-1}, positive-ion FAB-MS: *m/z* 481 $(M+Na)^+$) were superimposable on those of **1**, except for the signals due to the 3-hydroxyl group. Reduction of **2** with $NaBH_4$ yielded **1**. This evidence allowed us to conclude that the structure of myrrhanone A (**2**) is (5*S*,8*R*,9*R*,10*S*)-3-oxo-8,30-dihydroxypolypoda-13*E*,17*E*,21*E*-triene.



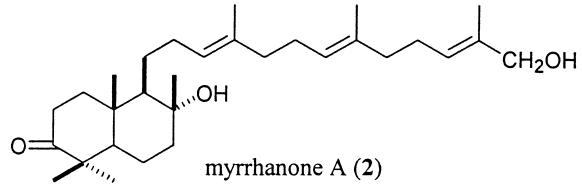


Chart 1.

Table 1. ^{13}C NMR data for myrrhanol A (**1**) and myrrhanone A (**2**)[a]

	1	2		1	2		1	2
C-1	37.9	38.3	C-11	25.5	25.8	C-21	125.7	125.7
C-2	27.0	33.9	C-12	31.3	31.2	C-22	134.7	134.6
C-3	78.6	217.0	C-13	125.1	124.8	C-23	28.1	26.3
C-4	38.8	47.4	C-14	135.0	135.1	C-24	15.4	21.3
C-5	55.0	55.1	C-15	39.6	39.6	C-25	15.5	14.8
C-6	20.2	21.3	C-16	26.5	26.5	C-26	23.7	23.5
C-7	44.3	43.7	C-17	124.5	124.4	C-27	16.2	16.2
C-8	73.9	73.6	C-18	134.6	134.6	C-28	16.0	16.0
C-9	61.1	60.3	C-19	39.3	39.3	C-29	13.7	13.7
C-10	38.8	38.5	C-20	26.1	26.1	C-30	68.6	68.6

[a]**1**: 125 MHz; **2**: 68 MHz, $CDCl_3$.

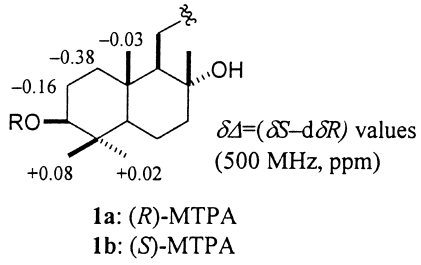


Figure 2. Modified Mosher's method for **1**.

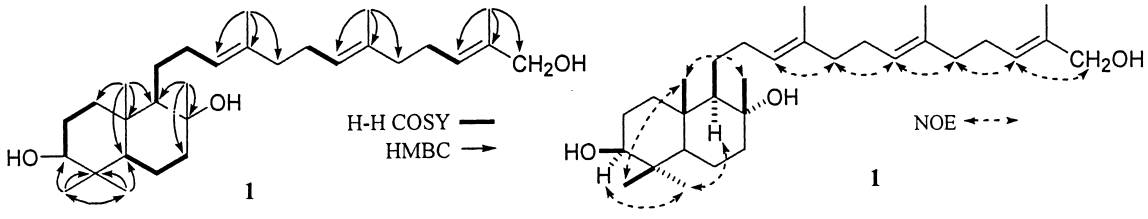


Figure 1. HMBC and NOE correlations of myrrhanol A (**1**).

Effects on inflammatory processes

Inhibitory effects on inflammatory processes I (exudation: pouch fluid weight), II (migration: inflammatory cell count), III (angiogenesis: carmine content), and IV (granuloma formation) were compared by using adjuvant-induced air pouch granuloma of mice as a model. Myrrhanol A (**1**) (0.6, 1.2, and 2.4 mg/kg), myrrhanone A (**2**) (0.6 mg/kg), guggul, and myrrh crude extracts (with 50% aqueous methanol and 100% methanol) (1.2, 6, and 30 mg/kg), and hydrocortisone (3.8, 7.6, and 15 mg/kg) were injected intraperitoneally, as described in the experimental sections. The dose–inhibitory response curves of myrrhanol A (**1**) and myrrhanone A (**2**) in regard to four different inflammatory parameters were compared with those of hydrocortisone (Fig. 3a). Myrrhanol A (**1**) was more potent than hydrocortisone, except for its inhibitory effect on the inflammatory cell count. Myrrhanone A (**2**) tended to be more effective than myrrhanol A (**1**) at 1.3 nmol/kg. The dose–response curves of myrrhanone A (**2**) were not obtained because of the less homogeneous suspensions at higher concentrations. The 50% inhibitory dose of myrrhanol A (**1**) was 2.80 μmol/kg (95% confidence limit: 2.07–3.79) for carmine content, 5.83 μmol/kg (3.03–11.2) for granuloma weight, and 1.75 μmol/kg (1.34–2.29) for

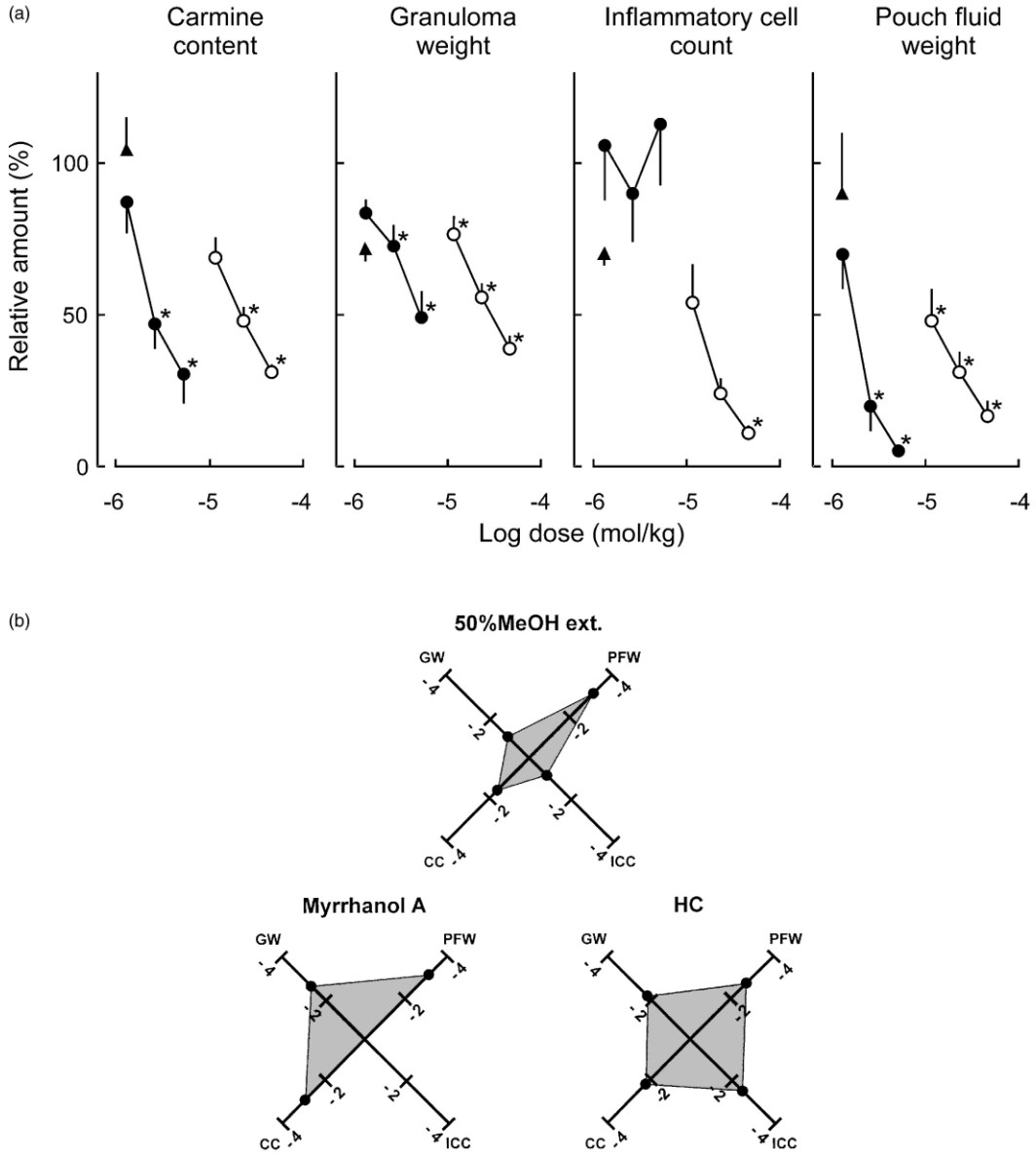


Figure 3. Potent anti-inflammatory effects of myrrhanol A (**1**) (closed circles, $n=4$–10) and myrrhanone A (closed triangles, $n=4$–12) derived from guggul-gum resin compared with those of hydrocortisone (HC) (open circles, $n=4$–12) on adjuvant-induced air-pouch granulomas of mice. The dose–effect curves on carmine content (CC), granuloma weight (GW), migrating inflammatory cell count (ICC), and exudative pouch fluid weight (PFW) (a) are plotted. The values represent the means ± SEM of relative values (%) calculated from the equation: (value with the compound)/(value without the compound) ×100 per mol/kg (injected intraperitoneally). The control values without the compound were 0.273 ± 0.022 mg for carmine content, 263 ± 20 mg for granuloma weight, (1.88 ± 0.21)×10^7 for cell count, and 198 ± 38 mg for pouch fluid weight ($n=19$). Log 50% inhibitory doses (ID_{50}, g/kg) of myrrhanol A (**1**) and 50% aqueous methanol extract of guggul-gum resin compared with those of HC for CC, GW, ICC, and PFW were estimated from the data in Figures 3a and 4, and are plotted on intersected axes (b). Points further from the origin 0 indicate more potent effects. *$P < 0.05$: Significant difference from control values without compounds.

pouch fluid weight, and the respective doses of hydrocortisone were 22.1 μmol/kg (18.0–27.1) for carmine content, 29.2 μmol/kg (23.1–36.9) for granuloma weight, 11.7 μmol/kg (7.15–19.0) for inflammatory cell count, and 6.31 μmol/kg (2.33–17.1) for pouch fluid weight. Myrrhanol A (**1**) had a 7.9, 5.0, and 3.6 times more potent effect on carmine content, on granuloma weight, and on pouch fluid weight, respectively, than hydrocortisone.

The 50% inhibitory doses (g/kg) of myrrhanol A (**1**), the 50% aqueous methanolic extract, and hydrocortisone were plotted on graphs of their relative effects on inflammatory response parameters (Fig. 3b). Myrrhanol A (**1**) was more potent than the 50% aqueous methanol extract on carmine content, granuloma weight, and pouch fluid weight, and the potency of myrrhanol A (**1**) than that of the 50% aqueous methanol extract was 23, 34 times greater, respectively. The 50% aqueous methanol extract, but not myrrhanol A (**1**), exerted weak effects on the inflammatory cell count.

The anti-inflammatory effects of crude extracts of guggul prepared with 50% aqueous methanol and with 100% methanol (Fig. 4) were compared with the effects of crude extracts of myrrh prepared with the same

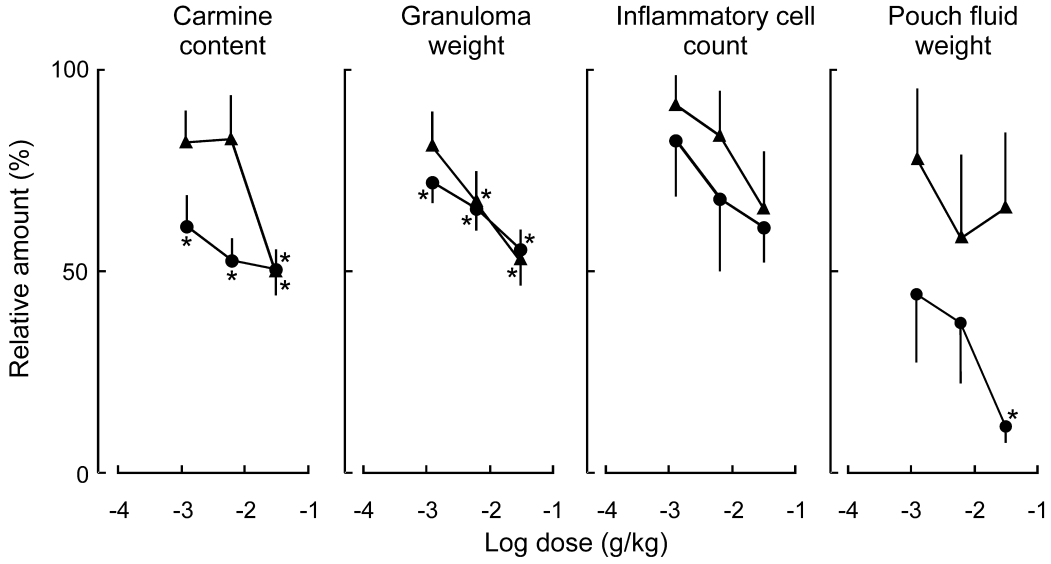


Figure 4. Anti-inflammatory effect of extracts of guggul-gum resin prepared with 50% aqueous methanol (closed circles, $n = 8$–13) and with 100% methanol (closed triangles, $n = 8$–12) in adjuvant-induced air-pouch granuloma of mice. Effects on carmine content, granuloma weight, migrating inflammatory cell count, and exudative pouch fluid weight are plotted. The values represent means ± SEM of relative value (%) calculated from the equation: (value with extract)/(value without extract) × 100 per g/kg (injected intraperitoneally). $*P < 0.05$: significant difference from control values without extracts.

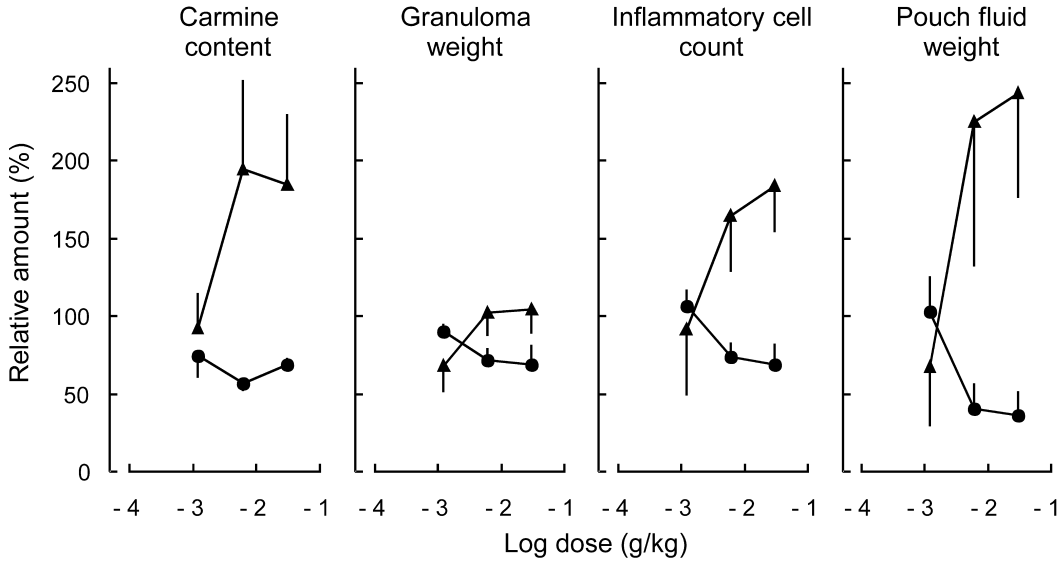


Figure 5. Weak anti-inflammatory and inflammatory effects of extracts of myrrh-gum resin prepared with 50% aqueous methanol (closed circles, $n = 5$–10) and with 100% methanol (closed triangles, $n = 4$–7) in adjuvant-induced air-pouch granulomas of mice. Effects on carmine content, granuloma weight, migrating inflammatory cell count, and exudative pouch fluid weight in mouse air-pouch granuloma are plotted. The values represent the means ± SEM of relative value (%) calculated from the equation (value with extract)/(value without extract) × 100 per g/kg (injected intraperitoneally). $*P < 0.05$: Significant difference from control values without extracts.

solvents (Fig. 5). The effects of the extracts of guggul tended to be more potent when prepared with a polar solvent than a less-polar solvent, especially on carmine content and pouch fluid weight (Fig. 4). The decoction of crude extracts of myrrh with the less-polar solvent instead tended to exacerbate the inflammation (Fig. 5). The anti-inflammatory activity of guggul gum is reported by using petroleum ether extract,[7] the steroidal fraction,[8] and steam distillate.[9] Myrrhanol A (**1**), a non-steroidal and hydrophilic compound, is more potent and may have fewer side effects than hydrocortisone.

In conclusion, myrrhanol A (**1**), a new compound isolated and identified from guggul-gum resin, is a plausible candidate for a potent anti-inflammatory agent.

Bioassay Methods

Crude drugs and agents

Hydrocortisone acetate was purchased from Nacalai Tesque (Kyoto, Japan). Myrrhanol A (**1**), myrrhanone A (**2**), and guggul and myrrh extracts (50% aqueous methanol and 100% methanol) (0.6–30 mg/kg) were solubilized or suspended homogeneously in saline containing 1% Avicel (Asahi Chemical Industry, Tokyo, Japan) and intraperitoneally injected 2 h after FCA (Freund's complete adjuvant) injection, then subsequently once a day for 4 days.

Animals

Male ddY mice (6 weeks of age, weighing 25.5–35.5 g) were purchased from Kiwa Laboratory Animal Science Co., Ltd (Wakayama, Japan). The mice were maintained under a constant temperature ($25\pm1\,^{\circ}C$) and humidity ($55\pm5\%$) with lights on from 7 a.m. to 7 p.m., and they were given free access to the usual laboratory diet (PMI Lab Diet, Japan Shizuoka Laboratory Center, Shizuoka, Japan) and tap water.

Adjuvant-induced air-pouch granuloma

Air-pouch granulomas were induced by injection of FCA with 0.1% croton oil, as reported previously.[10–12] The FCA emulsion was prepared by using 2 mg heat-killed *M. tuberculosis* (H37 RA, Difco, Detroit, MI, USA) per mL of Freund's incomplete adjuvant containing 0.1% croton oil (Nacalai Tesque), 42.5% liquid paraffin, 7.5% mannide monooleate (Nacalai Tesque), and 50% saline. Regular oval air pouches were produced by subcutaneously injecting 3 mL of air into the dorsum of the mouse under ether anesthesia. After 24 h, the FCA emulsion (0.5 mL) was injected into the air pouch under ether anesthesia. On day 5 after FCA injection, mice were killed by injection into the tail vein of 1 mL of 10% carmine solution (Merck, Darmstadt, Germany) containing 5% gelatin (Nacalai Tesque) warmed to 40 °C, and the dead mice were cooled to below 4 °C for several hours. The carmine content of the granuloma tissue was measured as follows. The granuloma tissue was cut, solubilized with 3 M NaOH, and acidified with 36% HCl. After centrifugation, the supernatant was filtered, and the carmine content of the filtrate, an index of newly formed blood vessels in the pouch granuloma,[7] was determined by measuring optical density at 490 nm. The granuloma tissue was excised, separated from the surrounding loose connective tissue, and weighed. The inflammatory cells in the pouch were counted with a hemocytometer. Granulocytes, monocytes and macrophages were included in the counting. Dead cells stained with trypane blue, erythrocytes and platelets were excluded. All the exudative pouch fluid was harvested and weighed.

Statistical analysis

Data are expressed as means $\pm$ SEM, and were analyzed by one-way analysis of variance, and then by Student–Newman–Keuls or Dunn's test at $P=0.05$ or $P=0.01$.

Acknowledgements

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