Volume 287, number 1,2, 97–101 FEBS 09987 August 1991

ADONIS 001457939100702P

Magnetic resonance studies of murine macrophages

Proliferation is not a prerequisite for acquisition of an 'activated' high resolution spectrum

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Received 21 March 1991; revised version received 28 May 1991

Proton magnetic resonance spectroscopy (^{1}H-MRS) was used to investigate the membranes of macrophages activated by γ-interferon in vitro and by *Listeria monocytogenes* in vivo. We report the appearance with activation, of a high resolution spectrum indistinguishable from that found in activated T and B cells and embryonic and malignant cell types previously studied. We furthermore show that proliferation is not a prerequisite for the appearance of this activated spectrum. This supports the idea that membrane 'activation' in all cells, irrespective of origin, may be accompanied by similar architectural changes, and suggests that a common pathway exists for the activation of cell membranes of the immune system, possibly important in the acquisition of increased motility. The use of ^{1}H-MRS as a non-invasive tool for analysis of activation is discussed.

NMR, ^{1}H-; Macrophage; IFN-γ; Activation

1. INTRODUCTION

There are few techniques available to biologists which have the non-invasive and immediate qualities of light microscopy and at the same time provide complex biochemical information. Proton magnetic resonance spectroscopy (^{1}H-MRS) has this capacity and we have used it in recent years to investigate the membranes of living cells [1,2]. An important feature of MRS is that molecules with very restricted motion, such as bilayer lipids and membrane proteins, are not detected under the conditions used to acquire high resolution spectra and this greatly simplifies the data obtained from living tissue. The information gained by this technique thus complements that obtained by other investigative methods and has shed new light on membrane architecture.

Certain cells, notably embryonic and some malignant types, exhibit a ^{1}H-MR spectrum dominated by high resolution lipid signals which arise from mobile neutral lipids in lipoprotein-like domains associated with the plasma membrane [3,4]. This spectrum also develops in T cells stimulated polyclonally by mitogens such as concanavalin A (Con A), or activated in mixed lymphocyte culture, while their normal resting counterparts exhibit a relatively featureless spectrum [5]. Spectral changes associated with activation of T cells are accompanied by up to 10-fold increases in cell membrane triglycerides and cholesteryl esters, similar to those found in malignant cells [3].

These cell types have in common a high degree of proliferation and a generally increased capacity for motility. This prompted us to investigate macrophages, which like T cells, may also be activated to be cytocidal and cytokine-secreting, but which are otherwise different, both functionally and morphologically. Macrophages are immunologically non-specific, and compared with T cells, are more primitive, developing in the bone marrow along a pathway different from that of T cells. Nevertheless, they may be activated to be strongly bactericidal and cytocidal by γ-interferon (IFN-γ) [6,7] produced by specifically activated T cells [8]. Indeed, the key factor in the normal in vivo eradication of intracellular pathogens such as *Listeria monocytogenes* is that of clearance by IFN-γ-activated macrophages [7].

In this study we report the appearance with activation, of a high resolution spectrum in macrophages, similar to that found in activated T cells. We show that cellular proliferation is not a prerequisite for the appearance of this activated spectrum. This supports the idea that membrane activation in cells, irrespective of origin, may be accompanied by similar architectural changes.

Abbreviations: ^{1}H-MRS, proton magnetic resonance spectroscopy; TSB, Trypticase Soy Broth; PBS, phosphate-buffered saline; SCC, standard culture conditions; MEM, Eagle's minimal essential medium; FCS, foetal calf serum; CRBC, chick red cells.

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2. MATERIALS AND METHODS

2.1. *Animals*

C57BL/6 mice obtained from the Blackburn Animal Breeding Establishment, University of Sydney, were used for all experiments.

2.2. *Listeria monocytogenes*

Virulent *L. monocytogenes* was cultured for 16 h in Trypticase Soy Broth (TSB) at 37°C. At this time 1 ml was removed and used to reseed a 10 ml fresh TSB and this was further incubated for 1 h at 37°C to allow the organisms to attain a log phase of growth. The concentration of organisms was determined by absorbance in a spectrophotometer set at 700 nm from a plotted standard curve.

2.3. *Interferon-γ*

Purified recombinant murine γ-interferon (IFN-γ) was a generous gift from Boehringer Ingelheim. Units stated are those given by them.

2.4. *Activation of macrophages*

A non-lethal dose of 10^6 virulent log phase *L. monocytogenes* was injected intraperitoneally into individual mice. Activated macrophages were recovered 72 h later by peritoneal lavage with cold phosphate-buffered saline (PBS). In vitro macrophage activation was achieved by incubation of resting macrophages, isolated by peritoneal PBS lavage, with 500 U/ml purified recombinant IFN-γ (Boehringer Ingelheim) at a concentration of 5×10^5/ml, under standard culture conditions (SCC), i.e. in Eagle's minimal essential medium (Gibco) supplemented with 5% foetal calf serum, antibiotics and 10^{-4} M 2-mercaptoethanol (MEM/FCS), in a humidified atmosphere of 5% CO_2 in air at 37°C.

2.5. *Preparation of cells for MRS*

Macrophages isolated from animals just prior to MRS were washed thoroughly in PBS prepared with D_2O to reduce the proton signal from water in the sample. In vitro treated macrophages were removed from the culture vessel with a rubber policeman. Aliquots of 10^8 cells were suspended in 400 μl PBS/D_2O and transferred to a 5 mm MRS tube for each experiment.

2.6. *MRS experiments*

^{1}H-MR spectra were recorded at 37°C using a Bruker WM400 and AM500 spectrometer. Peaks were referenced to sodium 3-(trimethylsilyl)propane-sulfonate. Two-dimensional correlated COSY spectra were recorded with a modified pulse sequence [9].

2.7. *BudR quenching of Hoechst 3342*

Resting peritoneal macrophages treated as above with and without IFN-γ were also subjected to the following protocol to determine the rate, if any, of cell division with activation in vitro. After 24 h, BudR medium (BudR 10 μg/ml, deoxycytidine 8 μg/ml in MEM/FCS) was added and the cells incubated as before, with and without IFN-γ, for a further 24 h under SCC. After this time, the cells were removed from the culture vessel and resuspended in MEM/FCS at a concentration of 5×10^5 cells/ml. To 1 ml of cells was added a pretitrated number of chick red cells (CRBC) and 50 μl of a solution of Hoechst 33342 (bisbenzimide trihydrochloride, Sigma , Cat no. B-2261) 200 μg/ml, and Nonidet P40, 0.1 ml in 9.9 ml MEM. After 30 min, stained nuclei were analysed by flow cytometry on a FACS IV (Becton Dickinson) with the laser set in UV at 30 mW. Emitted fluorescence was detected at >460 nm. Profiles were obtained on aliquots of 10^4 cells/sample.

3. RESULTS

3.1. *Comparison of resting and activated macrophages*

The ^{1}H-MR spectrum of resting macrophages isolated from the peritoneum by cold saline lavage is shown in Fig. 1A. The features of this spectrum are rather broad resonances, characteristic of protein with restricted motion, from which little structural information can be obtained. In contrast, macrophages activated in vivo with *L. monocytogenes* (Fig. 1B) display high resolution signals which can be assigned to lipids in a mobile environment, the intense peak at 1.3 ppm arising from methylene protons in lipid acyl chains. These results are very similar to those obtained with resting and stimulated lymphocytes [5]. ^{1}H-MRS of 10^9 *L. monocytogenes* showed no significant contributions to the regions of interest described here (data not shown) and there were far fewer organisms than this in our preparations.

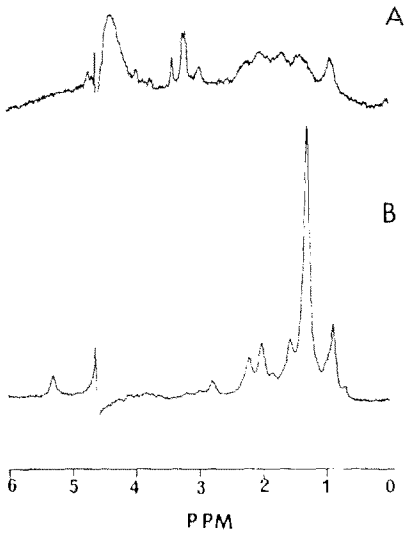
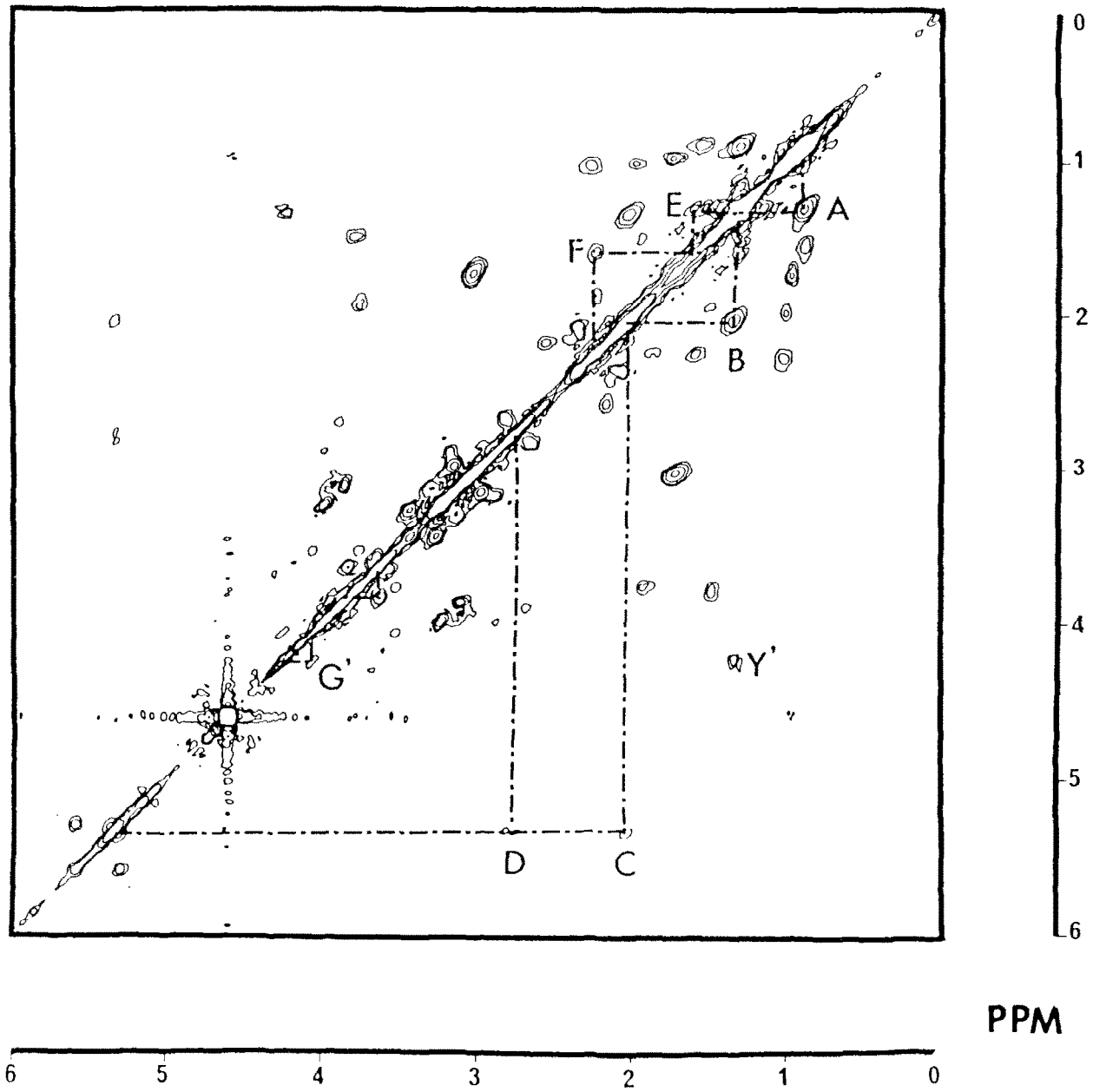


Fig. 1. One-dimensional ^{1}H-MR spectra of macrophages (1×10^8) suspended in PBS/D20. Spectra were collected at 37°C with the sample spinning and suppression of the residual HOD peak by gated irradiation. A line broadening of 3 Hz was applied. No vortex plug was used. A repetition time of 2 s was used and 64 transients were collected. The residual HOD resonance is observed at 4.7–4.8 ppm. A. Resting peritoneal macrophages. B. Peritoneal macrophages stimulated with *Listeria monocytogenes*.

Further information can be obtained from the 2D COSY spectrum shown in Fig. 2 where scalar coupling between non-exchangeable protons on adjacent atoms can be identified by the presence of symmetrically positioned cross-peaks joining resonances on the diagonal. Cross-peaks A–G′ have been assigned to triglyceride as shown in the structure. The other cross-peaks are from cellular metabolites. In particular, and Y′ is consistent with the methyl to methine couplings found in threonine and fucose and are also a feature of the MR spectra of activated lymphocytes [10] and malignant tissue [11]. Previous experiments with lymphocytes and malignant cells [3] indicate, that, rather than forming part of the more rigid bilayer, the triglyceride exists in a lipoprotein-like domain intercalated with the plasma membrane, within which it is free to tumble, giving rise to relatively narrow lines.



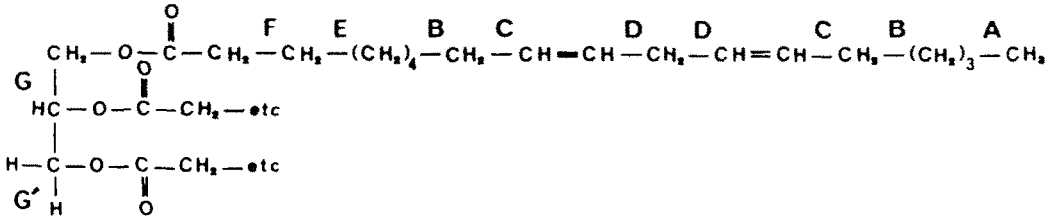


Fig. 2. Two-dimensional spectrum of 1×10^8 macrophages activated with *Listeria monocytogenes*. Spectra were collected at 37°C with the sample spinning and suppression of the residual HOD peak by gated irradiation. No vortex plug was used. A repetition time of 2 s was used and 32 transients were collected. The residual HOD resonance is observed at 4.7–4.8 ppm. Sinebell and Gaussian (LB = −30, GB = 0.22) window functions were applied in t_1 and t_2 domains, respectively. Lipid acyl chain and glycerol backbone connections (A–G) are as indicated in the structure.

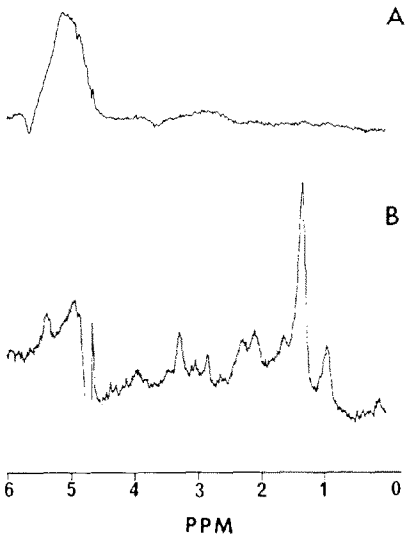


Fig. 3. One-dimensional ^{1}H-MR spectra of macrophages (2.5×10^7) suspended in PBS/D20. Spectra were collected at 37°C with the sample spinning and suppression of the residual HOD peak by gated irradiation. A line broadening of 3 Hz was applied. No vortex plug was used. A repetition time of 2 s was used and 1000 transients were collected. The residual HOD resonance is observed at 4.7–4.8 ppm. A. Untreated resting peritoneal macrophages cultured in vitro for 48 h. B. Peritoneal macrophages incubated with 500 U/ml purified recombinant γ-IFN in vitro for 48 h.

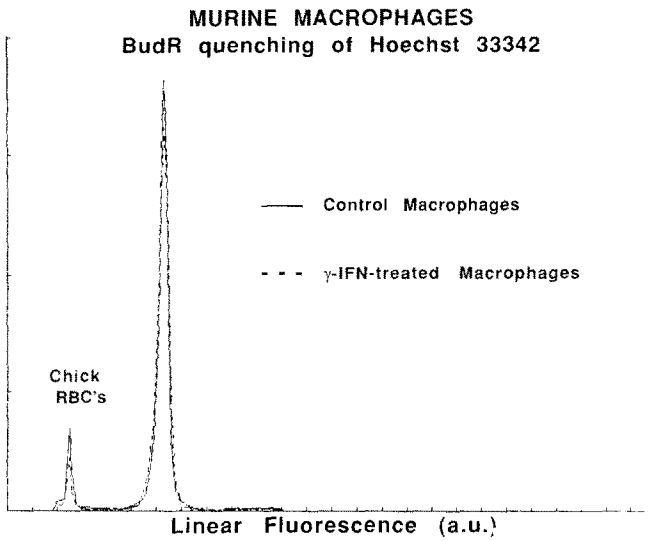


Fig. 4. Overlaid flow cytometric DNA histograms of in vitro cultured peritoneal macrophages stained with Hoechst 33342. Macrophages were treated with (---) and without (—) γ-IFN in vitro for 48 h. After 24 h, BudR was added and the cells incubated as before for a further 24 h. The cells were resuspended with a pretitrated number of chick red cells, Hoechst 33342 and Nonidet P40. Stained nuclei were analysed on a FACS IV with the laser in UV at 30 mW. Emitted fluorescence was detected at >460 nm. Profiles were overlaid to determine whether any displacement (signifying BudR quenching of Hoechst fluorescence and therefore proliferation) had occurred. No displacement of the histograms was seen. Controls, where BudR was not added, produced histograms identical to the above and were omitted for clarity.

3.2. *In vitro activation of resting macrophages*

The activation of macrophages to enhanced phagocytic and bactericidal capacity in vivo occurs via the production of IFN-γ by Listeria-specific immune T cells [6,7,12]. It was therefore of interest to know whether treatment in vitro with IFN-γ alone would result in the development of the activated high resolution spectrum seen in Listeria-activated macrophages in vivo. Peritoneal macrophages were allowed to adhere at non-confluent concentrations in culture flasks for 2 h before the supernatant containing non-adherent cells and debris was discarded and replaced with fresh medium. After a further 48 h they were removed with a rubber policeman for MRS examination. As shown in Fig. 3A, the MR spectrum is very similar to that in Fig. 1A for uncultured macrophages. The addition of IFN-γ to the resting macrophage cultures for 48 h resulted in the development of morphological characteristics of activated macrophages in vitro and of a lipid spectrum indistinguishable from that seen in macrophages activated by *L. monocytogenes* in vivo (Fig. 3B).

3.3. *Assessment of macrophage proliferation*

One of the common aspects of cells displaying a high resolution MR spectrum is rapid proliferation. In the case of the macrophages activated in vivo, recruitment is from the bone marrow where precursors proliferate and migrate to the peritoneal cavity [13]. However,with macrophages activated in vitro by IFN-γ, proliferation is not essential for activation. BudR is incorporated into proliferating cells and will quench Hoechst 33342 dye added subsequently. This technique was therefore used to assess cell cycling by flow cytometry. Macrophages were cultured for 24 h with and without IFN-γ and BudR was then added for a further 24 h. The quenching of Hoechst 33342 dye after this was measured by flow cytometry. No difference was observed between the histograms obtained from cells treated with IFN-γ and control cells (Fig. 4). In addition, no differences were seen between these cells and controls which had not been treated with BudR. Thus the IFN-γ-treated cells were either in G_0 or G_1 phase of the cell cycle despite the fact that they had developed a high resolution MR spectrum.

4. CONCLUSIONS

In this study we have investigated the MRS-characteristics of murine macrophages. We have demonstrated that macrophages activated by IFN-γ have membrane characteristics very similar to those of classically activated T cells and B cells as detected by ^{1}H-MRS. The high resolution lipid spectrum develops both in vitro and in vivo as a result of activation, but without this stimulus, under normal culture conditions this does not occur. This indicates that the phenomenon is not an in vitro artefact due to non-specific incorporation of lipid components from the culture medium.

One interpretation of this phenomenon is that a common pathway exists for the activation of membranes in cells of the immune system. That such a pathway may also be a feature common to other quite different cell types, as evidenced by the appearance of these characteristics both in malignant and embryonic cells,is of considerable interest.

We have also demonstrated conclusively that the development of these membrane characteristics does not depend on cellular proliferation, although it is frequently associated with it. Apart from proliferation, the cells displaying this activated spectrum have in common a generally increased motility. The question arising from this is whether membrane changes associated with activation are important in the acquisition of increased motility. This is currently being investigated.

Finally, we feel this technique has enormous potential as a quick, reliable and non-invasive method of assessment of the status of activating cells in the immune system [5,10] and while presently not widely used by biologists, holds promise as a tool for the analysis of early activation events in immune cells.

Acknowledgements: This work was funded by grants from the University of Sydney Cancer Research Fund and the Leo and Jenny Foundation.

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