Inhibition of mitogen-activated proliferation of human peripheral lymphocytes in vitro by propionic acid

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ABSTRACT

Recurrent infections are common features in patients affected by propionic acidaemia (McKusick 232000) and methylmalonic acidaemia (McKusick 251000). Since these disorders are biochemically characterized by tissue accumulation of propionic acid and methylmalonic acid respectively, it is possible that these compounds may act as immunosuppressants. We therefore investigated the effect of propionate and methylmalonate on cellular growth of human peripheral lymphocytes stimulated in vitro by phytohaemagglutinin, concanavalin A and pokeweed mitogen, a recognized test of cellular immunocompetence. Lymphocytes were cultured in flat-bottomed 96-well microplates at 37 °C for 96 h (phytohaemagglutinin and concanavalin A) or 144 h (pokeweed mitogen) in the presence of one mitogen at different concentrations and of one acid added at doses of 1.0, 2.5 or 5.0 mM. Cell blastogenesis was measured by the incorporation of tritiated thymidine into cellular DNA and compared with that of identical cultures with no acid added (controls). A consistent and progressive inhibitory effect of propionic acid with increasing concentrations in culture was identified with all mitogens and was more pronounced with pokeweed mitogen. Lymphocyte blastogenesis was not altered in the presence of methylmalonic acid. The effect of propionate was observed only when the drug was added at the beginning (phytohaemagglutinin-activated) or until 24 h (concanavalin A- and pokeweed mitogen-activated) of culture. The viability of lymphocytes after treatment with the drug, as assessed by the Trypan Blue exclusion test, revealed no change when compared with the same untreated lymphocytes, indicating no lymphocytotoxic activity. In conclusion, propionic acid, which accumulates in tissues of patients with propionic acidaemia, causes 'in vitro' immunosuppression, which may be related to the recurrent infections characteristic of these patients.

INTRODUCTION

Disorders in the metabolism of propionate (propionic acidaemia) and methylmalonate (methylmalonic acidaemia) are frequent diseases among the known organic acidaemias [1,2]. The two disturbances share various clinical and laboratory features. Propionic acidaemia is caused by deficiency in the activity of propionyl-CoA carboxylase (EC 6.4.1.3) and is biochemically characterized by greatly increased concent-
trations of free propionate in blood, and of propionate and some of its derivatives (methylcitrate, 3-hydroxypropionate and propionylglycine) in urine. Methylmalonic acidaemia is due to the deficient activity of t-methylmalonyl-CoA mutase (EC 5.4.99.2), leading primarily to the accumulation of methylmalonyl-CoA and secondarily of propionyl-CoA. Increased amounts of methylmalonate and of propionate in blood, as well as methylmalonate and some propionyl-CoA metabolites (methylcitrate and 3-hydroxypropionate) in the urine, are commonly found in these patients [3]. Among the many clinical and biochemical symptoms common to the two disorders, recurrent infections and neutropenia are observed in children affected by propionic acidemia and methylmalonic acidaemia [1,3–5]. Patients with propionic acidemia usually have respiratory tract or gastrointestinal infections, as well as a high frequency of skin lesions varying from nappy rash with *Candida albicans* infection to staphylococcal scalded skin syndrome, and some of them die of pneumonia and widespread infection [1,5,6].

Low resistance to infection may reflect immunosuppression and may be attributed to a variety of factors present in metabolic diseases such as malnutrition, acidosis and hypotonia. On the other hand, immunosuppressive activities have been found in a wide range of substances present in the serum of patients suffering from various pathological [7]. Therefore, it is possible that the toxic metabolites which accumulate in propionic acidemia and methylmalonic acidemia may act as immunosuppressants. The present study was undertaken to investigate the effect of propionate and methylmalonate on cellular growth of human peripheral lymphocytes stimulated in vitro by phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM), a well-recognized test of cellular immuno-competence, in order to identify the possible immuno-regulatory properties of these compounds.

**MATERIAL AND METHODS**

**Lymphocytes**

Peripheral venous blood samples from healthy adults were collected into heparinized tubes. The plasma was separated and the lymphocytes isolated by the Ficoll–Hypaque gradient method [8]. The cells were removed from the interphase and, after washing three times with 5% foetal calf serum (virus and mycoplasma screened, Gibco) in RPMI-1640 (with glutamine, Gibco), were centrifuged for 5 min at 400 g and finally suspended in RPMI-1640. The final concentration of the cell suspension was 2 x 10⁶ cells/ml medium. Cell viability, determined by the Trypan Blue test, was always higher than 95%.

**RESULTS**

The effects of propionate and methylmalonate, added separately to cultures at concentrations of 1.0, 2.5 and 5.0 mM, on PHA-, Con A- and PWM-induced lymphocyte response were studied (Figures 1–3). It can be seen that propionate inhibited lymphocyte proliferation whereas methylmalonate had no effect. Inhibition of PWM-stimulated lymphocytes was more accentuated than inhibition of lymphocytes treated with PHA or Con A. Increasing the concentration of the acid in culture provoked an additional inhibition of the rate of [*H*]thymidine incorporation.

Next, we added 5 mM propionate at different times during incubation and compared the lymphocyte responses to the mitogens. The inhibitory action of
Figure 1 Effect of propionic and methylmalonic acid on the ‘in vitro’ proliferative responses of human peripheral lymphocytes stimulated with PHA [0.25, 1.0 or 5% (v/v)].

Values are means and S.D. of eight experiments (lymphocyte donors) performed in triplicate. Differences between means of the various groups for each acid were calculated by ANOVA and by the Duncan multiple range test. Letters indicate significance between controls and the other groups; a, $P < 0.05$; b, $P < 0.01$.

Figure 2 Effect of propionic and methylmalonic acid on the ‘in vitro’ proliferative responses of human peripheral lymphocytes stimulated with Con A (12.5, 50 or 125 µg/ml).

Lymphocytes from four donors were used in the experiments. See Figure 1 for details.

Propionic acid occurred when the drug was added at the beginning of culture for PHA-stimulated lymphocytes or up to 24 h from the start of culture for cells stimulated with Con A and PWM (Figure 4).

Cell viability determined at the end of cultures supplemented by 5 mM propionate or methylmalonate was similar to that of control cultures, reflecting no cytotoxicity of the compounds tested (results not shown).

DISCUSSION

To our knowledge, little information is available in the literature about the effect of organic acids on the immune response. We previously reported that some acidic compounds accumulating in maple syrup urine disease have immunosuppressive properties [10]. The results presented in this paper demonstrate that propionic acid inhibits the transformation of human peripheral blood lymphocytes, as assessed by the incorporation of tritiated thymidine into cellular DNA. Our experimental system is generally accepted as one of the ‘in vitro’ models of ‘in vivo’ immune reaction [11]. Therefore, it is possible that the ‘in vitro’ immunoregulatory activities detected here may reflect the situation in vivo.

The concentrations of propionate added to cultures (1.0, 2.5 and 5.0 mM) correspond to those encountered in the blood of patients suffering from propionic acidemia. We believe that by cultivating human lymphocytes in
Figure 3 Effect of propionic and methylmalonic acid on the ‘in vitro’ proliferative responses of human peripheral lymphocytes stimulated with PWM [0.1, 0.25 or 1.0% (v/v)]

Lymphocytes from eight donors were used in this experiment. See Figure 1 for details.

![Graph showing effect of propionic and methylmalonic acid on lymphocyte proliferation](image)

Figure 4 Inhibitory effect of propionic acid (5 mM) added at different times during incubation on mitogen-induced lymphocyte proliferation

The values are expressed as percentage of lymphocyte reactivity (means ± I.D., n = 4) of control cultures (100% growth) where no acid was added. Differences between means of controls and the other groups were calculated by ANOVA followed by the Duncan multiple range test. Letters indicate significance between controls and the other groups. a, P < 0.05; b, P < 0.01.

![Graph showing inhibitory effect of propionic acid on lymphocyte proliferation](image)

vivo in the presence of propionate, we mimicked the situation in vivo.

We found that [³H]thymidine incorporation into stimulated lymphocytes was reduced by 2.5 and 5.0 mM propionate in PHA- and Con A-treated cultures, whereas in PWM-activated cultures this inhibition was more pronounced, occurring with doses of 1.0 mM and above. The effect of propionate was dose dependent since increasing the concentration of the acid in culture resulted in an augmented suppression.

The immunosuppression identified in vitro was not fortuitous, since it occurred at various doses of the drugs and of the mitogens. It was not a non-specific inhibition due to an acidic compound either, because methylmalonate, a structurally similar molecule, did not have any effect. Finally, it cannot be attributed to cytotoxicity since at the highest concentration used (5 mM) the acid did not decrease cell viability, as assessed by the Trypan Blue exclusion test.

Previous investigators have shown that patients with propionic acidaemia present a high incidence of infections [5,6]. Other authors observed ‘in vivo’ immunosuppression in these patients [12,13]. Muller et al. [12] found low serum IgG levels, leucopenia with lymphopenia and granulopenia, as well as depletion of T- and B-cells in lymph nodes from a child with propionic acidaemia. Lymphocytes from the patient responded well to various mitogens and antigens in the presence of 10% AB serum, but the same lymphocytes and those from normal individuals failed to respond when the culture medium contained 10% patients’ serum. These results indicate the presence of one or more immunosuppressive factors in the serum of affected patients, rather than a lack of growth factors. Furthermore, patients’ serum was not cytotoxic to lymphocytes since it did not decrease the viability of lymphocytes immersed in it for up to 72 h, as assessed by the Trypan Blue exclusion test. Similar findings were reported by Raby et al. [13] who described a patient with propionic acidaemia with low serum IgG and IgM levels, a normal proportion of T-cells and severe deficiency of peripheral B-cells.

The results reported in the present paper are in agreement with these studies and suggest that propionate may well be the suppressive factor or at least one of the immunosuppressants present in the serum of patients with propionic acidaemia.

In another report, it was verified that propionate strongly inhibits granulocyte/macrophage progenitor cell proliferation in marrow culture by approximately...
100% at concentrations of 3.2 mM, whereas methylmalonate had a mild inhibitory activity [14]. In addition, propionate did not reduce the viability of marrow cells in short-term cultures of 12-h duration. The authors propose that the neutropenia observed in patients with propionic acidaemia may be provoked by suppression of marrow neutrophil precursor proliferation by persistently high concentrations of the metabolite. They also suggest that neutropenia observed in patients with methylmalonic acidaemia may be a result of the concomitant increase of propionate or other proximal metabolites that occurs in this condition. Other investigators also found a clear dose-dependent propionate-mediated suppression of proliferation and maturation of haematopoietic progenitor cells and T-lymphocytic colony formation, and believe that the neutropenia, thrombopenia, anaemia and pancytopenia often observed during acute attacks in propionic acidaemia are due to increased tissue concentrations of propionate [15,16]. Therefore, it is possible that the immunosuppressive properties of propionate in vivo may be due to a combination of its effects on haemopoiesis, causing neutropenia and lymphopenia, and on the lymphocyte response, as demonstrated in the present work, which may explain at least in part the increased rate of infections in patients with propionic acidaemia.

The molecular mechanism by which propionate inhibits lymphocyte proliferation is unknown, but could be caused by impairment of energy production due to the inhibition of various enzymes by the acid [17,18].

In conclusion, the present data describe a biological activity of propionate, suggesting that this compound has an inhibitory effect on lymphocyte proliferation. Although our results should be interpreted cautiously, the 'in vivo' immunosuppressive activities already detected in some patients with propionic acidaemia strongly indicate that propionate is at least one of the factors responsible for such immunosuppression. Assessment of the reactivity of lymphocytes taken from patients with propionic acidaemia should also be a target for future studies in order to confirm our findings.

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REFERENCES


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