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Nicotinamide Adenine Dinucleotide Splitting Enzyme: A Characteristic of the Mouse Macrophage

Abstract. *Murine macrophages are endowed with nicotinamide adenine dinucleotide splitting activity that is markedly higher than that of other cells, tissues, or organs of the mouse. This enzyme therefore can be used as a biochemical marker for distinguishing macrophages from other cells of the lymphoreticular system and from polymorphonuclear leukocytes.*

In this report we present evidence to show that murine macrophages are endowed with NAD splitting activity (*1*) (NADase) that is much higher than that of other cells, tissues, and organs of the mouse. Moreover, within the elements of the biological defense system of the mouse, activity of this enzyme is confined almost entirely to macrophages. In contrast, polymorphonuclear leukocytes and T and B lymphocytes have very low or undetectable NAD splitting activity.

Since one of the problems in the preparation of macrophages for study is distinguishing them biochemically from lymphocytes and polymorphonuclear leukocytes, the presence of NADase can serve as a biochemical marker for murine macrophages.

Figure 1 illustrates the results of experiments on NADase activity of homogenates prepared from various mouse organs. All homogenates contained 500 μg of protein per milliliter, and the reaction was followed for 25 minutes at 37°C. Spleen had the highest activity, 110 units, and brain and heart the lowest, 15 and 7 units, respectively. Intermediate activity was found in lung (45 units), liver (36 units), and kidney (31 units) homogenates. These results are in agreement with reports of the high NADase activity of spleen (*2*).

To analyze the distribution of this enzyme among the various cells that make up the spleen, we fractionated splenic cells (*3*). The first step consisted of preparing purified nonadherent cells by filtering splenic cells through a glass wool column. This procedure removes macrophages and adherent subclasses of T and B lymphocytes.

Nonadherent cells (a mixed population of T and B lymphocytes and "null" cells) are practically devoid of NADase activity (Fig. 2). The removal of adherent cells similarly affected acid phosphatase (a lysosomal enzyme of monocytes and macrophages) and NADase activities. In some experiments, adherent cells were displaced from glass wool columns by squeezing the column with several 10-ml portions of phosphate-buffered saline without Ca^{2+} and Mg^{2+} until more than 95 percent of adherent cells were recovered. Splenic cells were also separated into adherent and nonadherent populations by plating them in petri dishes, with subsequent recovery of both types of cells. The results obtained confirmed our original finding that nonadherent cells were almost completely devoid of

Table 1. Evidence that NADase activity does not reside in adherent T or B lymphocytes. Activity was assayed in murine splenic and peritoneal cells depleted of T and B lymphocytes by complenent-dependent cytolysis. Nonfractionated and adherent splenic cells were prepared as described in the legend to Fig. 2. Peritoneal cells from noninduced mice were plated as described in the legend to Fig. 3 and used as monolayers. Cells (1×10^7/ml or per monolayer; ^{51}Cr-labeled, 40 minutes, 100 μCi/ml) in RPMI 1640 medium plus Hepes plus 0.3 percent BSA were incubated with antiserum to T cells (anti-T) or antiserum to mouse immunoglobulin (anti-B) at a final concentration of 1:10 for 60 minutes at 4°C, washed once in the same medium, and incubated with 1 ml of freshly reconstituted lyophilized rabbit complement diluted 1:10 in RPMI 1640-Hepes-BSA. After incubation for 45 minutes at 37°C the supernatants were aspirated and portions (0.1 ml) were counted in a well-type gamma counter. The lytic effect was determined by the formula:

$$\frac{\text{experimental } {}^{51}\text{Cr release} - \text{spontaneous release}}{\text{maximal release (freeze thaw)} - \text{spontaneous release}} \times 100$$

The sedimented splenic cells were brought to the original volume in 0.1*M* phosphate buffer, *p*H 7.2, and assayed. Peritoneal cells were assayed in the form of monolayers. Control cells (incubated without antiserum, or complement or both) were identically treated. The number of experiments is shown in parentheses.

Treatment	Cell lysis (%)	NAD split (μmole/25 minute)
Splenic cells, nonfractionated		
Control	< 2	0.31 ± 0.2 (6)
Anti-T + C	32	0.32 ± 0.3 (6)
Anti-B + C	21	0.33 ± 0.3 (6)
Splenic cells, adherent		
Control		0.42 ± 0.3 (6)
Anti-T + C		0.42 ± 0.4 (6)
Anti-B + C		0.43 ± 0.3 (6)
Peritoneal cells		
Control	0.2	0.36 ± 0.4 (4)
Anti-T + C	4.0	0.38 ± 0.4 (4)
Anti-B + C	3.5	0.37 ± 0.3 (4)

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NADase activity. The total activity of nonfractionated splenic cells could be accounted for by the activity of adherent cells.

The results of experiments summarized in Table 1 indicate that it is highly unlikely that NAD splitting activity of splenic cells resides in adherent subclasses of T and B lymphocytes. In these experiments, nonfractionated and adherent splenic cells were treated with antiserum to T cells or antiserum to mouse immunoglobulin, plus complement. The cytotoxic assay procedure with antiserum to T cells killed more than 95 percent of murine thymus cells and 32 percent of nonfractionated splenic cells. The antiserum to mouse immunoglobulin cytotoxic procedure killed approximately 21 percent of nonfractionated splenic cells. Depletion of splenic cells of T and B lymphocytes by complement-dependent cytolysis had no effect on total NADase activity of the spleen; that is, when, after cytotoxicity assay, the cells were adjusted to the original volume, NADase activity of control and that of experimental cell suspensions was identical. Our conclusion from these experiments was that spleen NADase resided in macrophages and not in adherent subclasses of T or B lymphocytes.

We therefore analyzed other lymphoid organs and their constituent cells for the presence of an enzyme involved in NAD degradation. Cell populations so tested were murine peritoneal and alveolar macrophages and thymus, bone marrow, and mesenteric lymph node lymphocytes, as well as human and murine peripheral blood monocytes, lymphocytes, and polymorphonuclear leukocytes (Fig. 3).

Mesenteric lymph node and bone marrow lymphocytes had no detectable NADase activity. Polymorphonuclear leukocytes and thymus and peripheral blood lymphocytes had very low activity, from 1 to 2 units per 5×10^6 cells. The activity of monocytes varied from 1 to a maximum of 20 units per 5×10^6 cells. Peritoneal and alveolar macrophages were the only cells with high and reproducible activity, about 100 units per

Table 2. NADase activity of human and murine erythrocytes and platelets. Red cells from freshly drawn, heparinized blood were sedimented and washed four times in ideal milliosmolar phosphate buffer, *p*H 7.2. Intact cells were used for the assay. Platelets were prepared as described (*15*). Peritoneal macrophages were prepared as described in the legend to Fig. 3. The reaction mixture contained red blood cells (70 mg of protein per milliliter); platelets (1 mg of protein per milliliter), or macrophages (250 μg of protein per milliliter). NADase was assayed by cyanide addition reaction (see legend to Fig. 1). The number of experiments is shown in parentheses.

Cells	NADase (units/ 500 μg protein)
Peritoneal macrophages	100.00 ± 4.0 (10)
Platelets (human)	11.20 ± 0.8 (4)
Platelets (murine)	12.10 ± 0.7 (7)
Erythrocytes (human)	0.10 ± 0.02 (7)
Erythrocytes (murine)	0.10 ± 0.03 (6)

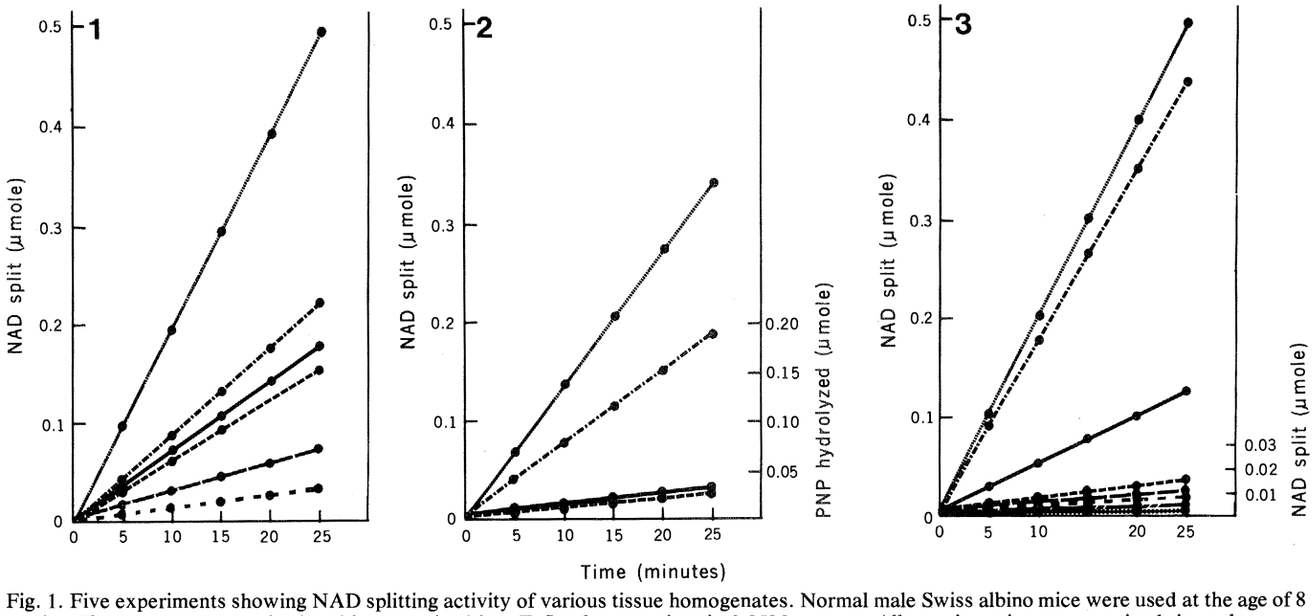

Fig. 1. Five experiments showing NAD splitting activity of various tissue homogenates. Normal male Swiss albino mice were used at the age of 8 weeks. The organs were excised and homogenized in a Teflon homogenizer in 0.25*M* sucrose. All reaction mixtures contained tissue homogenates, 500 μg of protein per milliliter in ideal milliosmolar phosphate buffer, *p*H 7.2, and 0.0006*M* NAD (Sigma). The reaction was followed by the cyanide procedure (*6*). A unit of enzyme is the amount that cleaves 1 nmole of NAD in 5 minutes at 37°C. Protein was determined by the method of Lowry *et al.* (*9*). Spleen, ; lungs, ; liver, ; kidneys, ; brain, ; and heart, . Fig. 2. Nine experiments showing NADase and acid phosphatase activity of splenic cells. The cells were separated into adherent and nonadherent cells (*3*) and assayed for NADase (see the legend to Fig. 1) and acid phosphatase (*10*). Results of acid phosphatase assays were plotted in terms of micromoles of *p*-nitrophenyl phosphate (*PNP*) hydrolyzed in unit time. All reaction mixtures contained 5×10^6 cells per milliliter. Nonfractionated splenic cells: acid phosphatase, ; NADase, . Nonadherent cells: acid phosphatase, and NADase. Fig. 3. NADase activity of thymus, mesenteric lymph node, and bone marrow lymphocytes, peritoneal and alveolar macrophages, and monocytes, lymphocytes and polymorphonuclear leukocytes from murine peripheral blood. Peritoneal macrophages were prepared from noninduced peritoneal cell suspensions plated in plastic petri dishes (*11*). Alveolar macrophages were obtained as described (*12*). Thymus, bone marrow, and mesenteric lymph node cells were prepared as described (*13*). Murine blood was obtained from 60 mice by cardiac puncture and fractionated by Ficoll-Hypaque gradient centrifugation (*14*). A band of mononuclear cells (lymphocytes and monocytes) was obtained at the interface between plasma and Ficoll, and a mixture of red blood cells and polymorphonuclear leukocytes sedimented to the bottom of the centrifuge tube. The mononuclear cells were separated into adherent cells (monocytes) and nonadherent cells (lymphocytes) by the same procedure used for splenic cells (see the legend to Fig. 2). Polymorphonuclear leukocytes were separated from red blood cells by dextran sedimentation. All preparations were adjusted to 5×10^6 cells in ideal milliosmolar phosphate buffer, *p*H 7.2. NADase activity was assayed as described in the legend to Fig. 1. Peritoneal macrophages, ; alveolar macrophages, ; monocytes, ; thymocytes, ; lymphocytes, ; polymorphonuclear leukocytes, ; bone marrow cells, ; and mesenteric lymph node cells, .

5×10^6 cells. Activity of peritoneal cells remained unchanged after the cells were treated with antiserum to T and antiserum to mouse immunoglobulin in the presence of complement (Table 1).

Results of experiments with human peripheral blood monocytes, lymphocytes, and polymorphonuclear leukocytes were similar to those obtained with the corresponding murine cells.

The other formed elements of peripheral blood assayed for NADase activity were erythrocytes and platelets (Table 2). Both human and murine red blood cells had negligible activity as compared with that of peritoneal, alveolar, and spleen macrophages—approximately 0.1 unit per 500 μg of erythrocyte protein, as compared to 100 units per 500 μg of macrophage protein. Thus, activity of red blood cells was approximately one-thousandth that of macrophages. Platelets, on the other hand, showed a relatively high activity, 11 to 12 units per 500 μg of protein, a value surpassed only by the activity of macrophages.

Platelets share several properties with macrophages, such as the property to adhere to foreign surfaces, and the capacity to engulf inert particles, which represents true phagocytosis. Although bactericidal and lytic properties have been ascribed to platelets, their antixenic function has not been proved (*4*).

Two or possibly three enzymes split NAD at the nicotinamide riboside linkage. The single protein NADase (E.C. 3.2.2.5) also has two activities: NAD glycohydrolase and NAD transglycosidase. The common intermediate is ADP ribosylated (ADPR) enzyme, which promotes transfer of the ADPR moiety of NAD to water (glycohydrolase activity) or to various pyridine derivatives in place of nicotinamide (transglycosidase activity). The two other enzymes are ADP-ribosyl transferase, which catalyzes the transfer of ADPR moiety of NAD to a macromolecular acceptor, and poly(ADPR) synthetase, which promotes polymerization of ADPR. The formation of poly(ADPR) seems to be restricted to eukaryotic nuclei having chromatin structure (*5*).

The activity of all three enzymes can be followed by the cyanide addition reaction (*6*). The quaternary nitrogen of the β-*N*-glycosidic linkage is essential for the reaction. When NAD is split at the pyrophosphate bond, there is no change in the cyanide reaction. Poly(ADPR) synthetase can be distinguished from NADase by the formation of an acid-precipitable ADPR polymer.

We do not yet know which of the above enzymes is responsible for the cleavage of NAD in murine macrophages. Throughout the text we have chosen to refer to the NAD splitting enzyme of macrophages and platelets as NADase, because the spleen enzyme was reported to have transglycosidase activity (*2*).

The biological function (or functions) of the enzymes which split NAD are unknown, with the notable exception of NAD glycohydrolase of toxigenic strains of *Corynebacterium diphtheriae*, where the enzyme was identified as the diphtheria toxin (*7*). The existing data on the possible role of NAD splitting enzymes in eukaryotes are both fragmentary and contradictory (*8*).

It would be of interest to ascertain whether the NAD splitting activity of macrophages (and platelets) is related to their function and whether the high NADase activity of lungs and liver, like that of spleen, is the property of lung and liver macrophages.

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References and Notes

1. Abbreviations used: NAD, nicotinamide adenine dinucleotide; ADPR, adenosine diphosphate ribose; poly(ADPR), polymer of adenosine diphosphate ribose; C, complement; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Antiserum to mouse immunoglobulin and antiserum to T cells (that is, to theta antigen) rabbit complement (absorbed with agarose and mouse tissues) were obtained from Cedarline Laboratory, London, Ontario, Canada, and from Miles Laboratory, Elkhart, Ind.
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Cell Lineage Analysis by Intracellular Injection of a Tracer Enzyme

Abstract. *Cell lineages during development of leeches can be ascertained by injection of horseradish peroxidase as a tracer into identified cells at early stages of embryogenesis. The injected embryos continue their normal development, in the course of which horseradish peroxidase is passed on in catalytically active form to the descendants of the injected cell. The distribution of the tracer enzyme and hence of the progeny of the injected cell can then be observed at a later stage of development by staining the preparation for horseradish peroxidase.*

Knowledge of the lines of descent of the cells that eventually compose the mature organism is fundamental for understanding embryonic development. Regulative mechanisms, such as differentiation controlled by position within an external gradient, and determinant mechanisms, such as differentiation controlled by inherited cytoplasmic factors, may be involved to varying degrees at different developmental stages; learning the fate of clonally related cells can aid in discerning the relative importance of these mechanisms.

In the past, cell lineages have been established by the direct observation of dividing cells or of extracellular marker particles, by selective ablation, and by induction of genetic mosaics (*1*). Each of these techniques has disadvantages that limit its scope: direct observation of living embryos becomes progressively more difficult as cells increase in number and decrease in size; ablation experiments are difficult to interpret because of the disruption of normal development; genetic mosaic studies are hampered by a paucity of suitable genetic markers and by the difficulty of preselecting the clonal progenitor.

We now present a new procedure for the determination of cell lineages, which extends the scope of cell lineage analysis by virtue of its precision and specificity. In this technique horseradish peroxidase (HRP) is used as an intracellular tracer, injected through a micropipette into identified cells of early embryos. After HRP injection, embryonic development is allowed to progress to a later stage, at which time the distribution pattern of HRP within the embryonic tissues is visualized by staining for its presence. The success of this method requires that at