Preparation and Characterization of Monoclonal Antibodies to a *Butyrivibrio* sp. and Their Potential Use in the Identification of Rumen Butyrivibrios, Using an Enzyme-linked Immunosorbent Assay

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(Received 15 May 1985; revised 22 July 1985)

Antisera and monoclonal antibodies (McAb) were prepared against the proteolytic rumen bacterium *Butyrivibrio* strain PI-7, and their reaction with bacterial antigens was quantified by using an enzyme-linked immunosorbent assay. A dye-release assay was developed to assess the binding of fixed bacterial cells to microtitration plates. Five McAb were screened against 15 type cultures representing other important rumen genera, five type cultures of the genus *Butyrivibrio* and 63 presumptive butyrivibrios, which were assigned to the genus on the basis of their biochemical and morphological characteristics. Antiserum was also screened against all strains of *Butyrivibrio*. Of all the bacteria tested, only six presumptive butyrivibrios cross-reacted with two or more McAb; six others cross-reacted with at least one McAb; 20 butyrivibrios, including three of the type cultures, cross-reacted with the antiserum. Binding-competition studies indicated that the McAb were directed against three different antigenic determinants. It is concluded that McAb have potential uses in grouping butyrivibrios and as a specific probe for a single strain.

INTRODUCTION

Members of the genus *Butyrivibrio* are among the most numerous and biochemically versatile rumen bacteria. They have been isolated from the forestomachs of different species of ruminants under a range of feeding regimes (Hungate, 1966; Roché *et al.*, 1973) and are involved in the ruminal digestion of cellulose (Roché *et al.*, 1973), the hydrolysis of dietary lipid (Hazlewood & Dawson, 1975, 1979), the hydrogenation of polyunsaturated fatty acids (Polan *et al.*, 1964) and in the production of butyrate and the interconversion of acetate and butyrate (Latham & Legakis, 1976; Van Gylswyk, 1976). Butyrivibrios are heterogeneous with respect to substrates fermented, fermentation products (Bryant & Small, 1956; Shane *et al.*, 1969), nutritional characteristics (Bryant & Robinson, 1962; Shane *et al.*, 1969), and serology (Margherita & Hungate, 1963; Margherita *et al.*, 1964). A superficial subdivision of strains, based on nutritional requirements and the pattern of production of fermentation acids has been proposed (Shane *et al.*, 1969), but no definitive taxonomic studies have been done. Currently there appears to be no basis for subdivision of the genus and if species other than the type species *B. fibrisolvens* exist, biochemical characterization alone does not provide a basis for their definition.

The potential application and use of antisera and immunofluorescence microscopy for the serological analysis of rumen micro-organisms has been investigated by a number of authors.

Abbreviations: FCS, foetal calf serum; McAb, monoclonal antibodies.

Serological techniques showed that different morphological types of rumen bacteria, observed *in vivo*, were related to pure cultures of known micro-organisms cultured *in vitro* (Hobson *et al.*, 1955, 1958, 1962; Hobson & Mann, 1957). Serological evidence also led Jarvis (1967) to suggest the existence of two different species within the genus *Ruminococcus*, and enabled him and his co-workers to observe diurnal variations in ruminal numbers of cellulolytic cocci by using fluorescent antisera (Jarvis *et al.*, 1967). Similarly, different serotypes were shown to exist in the genus *Butyrivibrio*, but within this large and heterogeneous group it was not possible to define taxa on the basis of antigenic relatedness (Margherita & Hungate, 1963; Margherita *et al.*, 1964). More recently, both antisera and McAb have been used to establish antigenic relationships and a rapid identification procedure for the methanogenic archaeobacteria, some species of which comprise part of the normal rumen microflora (Conway de Macario *et al.*, 1982a, b, c; Macario & Conway de Macario, 1982).

In this paper we describe the production and characteristics of McAb raised against a rumen bacterium assigned to the genus *Butyrivibrio*. In undertaking the study, we had two principal objectives in mind. Firstly, to investigate whether, within a large and heterogeneous genus like *Butyrivibrio*, McAb could form the basis of a rapid identification procedure, enabling bacteria to be assigned to well-defined groups according to antigenic relatedness, and secondly, to determine whether such McAb would possess the specificity to permit subsequent identification and enumeration of selected species in natural populations, or mixed laboratory cultures.

METHODS

Rumen bacteria and culture conditions. Type cultures (15) representing the major genera of rumen bacteria were obtained from the National Collection of Dairy Organisms, from culture collections at the Animal and Grassland Research Institute and the Institute of Animal Physiology and from colleagues. Presumptive *Butyrivibrio* spp., including the proteolytic strain PI-7 which was used to raise antiserum and McAb, were isolated from cows (Hazlewood *et al.*, 1983), seaweed-eating Orkney sheep (Greenwood *et al.*, 1983) and Svalbard reindeer (Orpin *et al.*, 1985). All rumen bacteria were cultured at 39 °C in medium B, a semi-defined rumen fluid-free liquid medium (Lowe *et al.*, 1985), by aseptic anaerobic techniques (Miller & Wolin, 1974; Ogimoto & Imai, 1981).

Overnight cultures (10 ml) of the type species representing the major genera of rumen bacteria and of the *Butyrivibrio* strain PI-7 were used to inoculate 100 ml of pre-warmed medium in a gas phase of O₂-free CO₂ in 125 ml serum bottles (Phase Separation Ltd). Cells were harvested by centrifugation (3000 g, 15 min, 4 °C) in the late exponential phase of growth. Presumptive butyrivibrios, other than strain PI-7, were cultured in medium B (9.5 ml medium B plus 0.5 ml inoculum) in screw-capped anaerobic culture tubes (Bellco Glass Inc.) and harvested as described above. Harvested cells were fixed by resuspending pellets in aqueous 0.85% NaCl solution containing 1.6% (w/v) formaldehyde (Conway de Macario *et al.*, 1982b) to an OD₆₅₀ of 0.5 (Spectronic 20 spectrophotometer; Bausch & Lomb). Suspensions of fixed bacteria were stored at 4 °C.

A comparison of the ability of presumptive butyrivibrios and type cultures of the genus to ferment different carbon sources was made in liquid medium B to which carbon sources (0.4% final concentration) had been added according to the schemes of Hungate (1966) and Ogimoto & Imai (1981). Growth was assessed either by measuring culture turbidity after 1, 2 and 5 d incubation, or by comparing the concentration of volatile fatty acids in the culture supernatant with the concentration in uninoculated medium after 5 d incubation. Volatile fatty acids were determined as described by Lowe *et al.* (1985) and were used to assess growth on insoluble or optically opaque substrates such as amygdalin, glycogen, starch and xylan.

Binding of cells to micro-ELISA plates. A colorimetric procedure for determining whether fixed bacterial cells bind to micro-ELISA plates was based on the affinity of the dye PAGE blue G90 (BDH) for proteins (Bramhall *et al.*, 1969). Plates were coated with bacterial cells and washed with phosphate-buffered saline (PBS) (NaCl, 0.85 g l⁻¹; Na₂HPO₄, 1.07 g l⁻¹; NaH₂PO₄, 0.039 g l⁻¹; NaN₃, 0.5 g l⁻¹) containing 0.15% Tween 20 (PBST). PAGE blue G90 (100 µl of 0.5 g l⁻¹ in 7% v/v acetic acid) was added to each well and the plate was shaken for 15 min at room temperature (setting 3; Titertek plate shaker; Flow Laboratories). Excess dye was removed from each well by washing six times with 7% (v/v) acetic acid. Dye bound to protein contained in the bacterial film was released by adding 100 µl of methanol/water/conc. ammonia (66:3:1, by vol.) to each well and shaking the plate at room temperature for 15 min. Colour liberated was measured at 620 nm (Multiscan MC plate reader; Flow Laboratories).

Preparation of antisera and hybrid myelomas and screening by ELISA. Fixed cells of *Butyrivibrio* PI-7 from 2 ml cell suspension (OD₆₅₀ 0.5) were washed and resuspended in 0.6 ml PBS, prepared as described above but without the addition of Tween 20. After emulsifying in an equal volume of complete Freund's adjuvant, 200 µl samples of bacterial suspension were injected intra-muscularly into three female rats (strains DA, PVG and F344). Each rat

was boosted at 2 month intervals with a subcutaneous injection of bacteria prepared as above, but in incomplete Freund's adjuvant. Test bleeds taken 10 d after the third boost were found by ELISA to contain antibodies. Six weeks later one of the rats (strain PVG) was injected intravenously with washed cells of strain PI-7 from 1 ml of fixed cell suspension resuspended in PBS (2 ml). The spleen was removed 3 d later, and the spleen cells were fused with cells of the rat myeloma line Y3 Agl. 2.3 (Galfre *et al.*, 1979), as described by Galfre & Milstein (1981). Then 14 d after fusion, supernatant fluids from confluent cultures were tested by ELISA for the presence of specific McAb. Selected positive cultures were cloned in soft agar and individual clones picked up when at the stage of a few hundred cells.

Culture supernatants or rat antisera were screened for the presence of homologous antibodies using a solid-phase ELISA, based on the quantitative immunoenzymic assay described for methanogens by Conway de Macario *et al.* (1982*b*), and similar in practice to the assay described by Archer (1984). A suspension of fixed bacterial cells (OD_{650} 0.5) containing 0.30 mg dry weight cells ml^{-1} was centrifuged (3000 g, 15 min, 4 °C) and the supernatant fluid was discarded. The pellet was routinely resuspended in five times the original volume of glass-distilled water and 0.1 ml samples were pipetted into 96-well micro-ELISA plates (M129B Immulon, Dynatech, or Falcon 3912 MicroTest III flexible plates, Becton Dickinson). Plates were left at 39 °C overnight during which time bacterial cells in the suspension dried on to the lower portion of the wall and the bottom of each well. When necessary, sensitized plates were wrapped in foil and stored at 4 °C in polythene bags until required; no deterioration was apparent after 14 d storage. Before use the bacterial film was washed three times with PBST and after blocking solution (0.15 ml), comprising 10% (v/v) foetal calf serum (FCS) in PBS, had been introduced into each well, the plate was incubated at 39 °C for 1 h. Each well was then washed three times with PBST as before, and antiserum or culture supernatant (0.1 ml), diluted where necessary with PBS containing 2% (v/v) FCS, was added to each well and plates were incubated at 39 °C for 1 h. After washing (three times with PBST), anti-rat IgG conjugated to horseradish peroxidase (Miles Laboratories) and diluted 1000 to 2000 times with PBS containing 2% (v/v) FCS was added (0.1 ml) to each well. Plates were incubated at 39 °C for 1 h. After washing (four times with PBST), chromogenic substrate (0.1 ml), prepared by mixing glass-distilled water (18 ml), 1 M-sodium acetate/citric acid buffer, pH 6.0 (2 ml), *N,N,N',N'*-tetramethylbenzidine ($10 g l^{-1}$) in DMSO (0.2 ml) and 6% (v/v) H_2O_2 (0.02 ml), was added to each well. Plates were incubated at room temperature for 10 to 20 min with continuous shaking and the reaction was terminated by adding 0.25 ml 3 M- H_2SO_4 . The absorbance of product formed in each well was measured at 450 nm (Multiscan MC plate reader). Wells to which antigen had been added, but which subsequently received pre-immune serum or uninoculated tissue culture medium, were included as blanks on all plates.

Radiolabelling of McAb and binding-competition assay. Cells from cloned lines were cultured in media containing [3H]lysine and the secreted radiolabelled McAb were analysed by SDS-PAGE after reduction with 2-mercaptoethanol (Kohler *et al.*, 1976; Laemmli & Favre, 1973). Before use in binding-competition assays, radioactive supernatants were dialysed against four changes of 100 vols of PBS.

The binding-competition assay evaluated the ability of a 3H -labelled McAb to bind to homologous antigen in the presence of varying concentrations of competing unlabelled McAb and was done in flexible 96-well PVC plates (Falcon 3912 MicroTest III) coated with fixed cells of *Butyrivibrio* PI-7. Remaining protein-binding sites were blocked and plates were washed as described previously. For each unlabelled McAb to be tested, serial dilutions in PBS containing 2% (v/v) FCS were prepared to span a 50-fold concentration range. Diluted McAb (90 μl) were mixed in a sensitized well with 10 μl 3H -labelled antibody containing 1.5 to 2.2×10^6 Bq and, after an initial period of shaking (3 min), were incubated statically at 39 °C for 1 h. After removal of the antibody solution, wells were washed four times with PBST and blotted dry. The wells were then cut from the plate using an electrically-heated wire (Galfre & Milstein, 1981), placed in scintillation vials and counted after the addition of 10 ml scintillant (4 g PPO, 0.4 g POPOP, 1 litre toluene). Control wells contained only 3H -labelled antibody in PBS with 2% (v/v) FCS, and no competing unlabelled McAb.

RESULTS

Enzyme-linked immunosorbent assay

The ELISA was initially developed for screening antiserum samples prepared by immunizing rats with formalin-fixed *Butyrivibrio* PI-7 cells. Colour development due to non-specific adsorption occurred with pre-immune serum when diluted fivefold, but was negligible at a dilution of 10^{-3} . In contrast, immune serum gave rise to appreciable colour development when diluted more than 10^{-4} (Fig. 1). In a typical assay, PVC wells were coated with 6 μg antigen (whole cells), and 100 μl antiserum (diluted 10^{-3} to 10^{-4}), or culture supernatant fluid containing McAb (diluted 25 to 50 times) was sufficient to give absorbance values in the region of 1.

Fig. 1

Fig. 2

Fig. 1. Detection of homologous antibodies by ELISA (as described in Methods). Immune sera (●, ○) were obtained from rats which had been injected four times with antigen over a period of 6.5 months. Normal serum (▲) was taken from a rat which had not been injected with antigen.

Fig. 2. Release of PAGE blue G90 by butyrvibrios coated on to microtitration plates. Plates were coated with strain PI-7 (●) or strain Bu49 (○), using cell suspensions of different concentrations. Each absorbance value for released dye is the mean of triplicate determinations.

To evaluate the ability of fixed cells of a number of different bacteria to cross-react with antiserum or McAb prepared against *Butyrvibrio* PI-7, it was necessary to determine first whether the cells of each test organism could bind to micro-ELISA plates. The uptake of PAGE blue G90 by the bacterial film and its subsequent release into the destaining solution proved in all cases to be a qualitative index of cell binding. For two butyrvibrios, strain PI-7 and strain Bu49, the uptake of dye and presumably the binding of cells to the PVC wells was comparable and almost linearly related to the concentration of cells in the sensitizing suspension (Fig. 2).

The ability of 10 different species of rumen bacteria to bind to the PVC wells was examined by using the dye-release assay. At a fixed coating concentration (twofold dilution of the working suspension), the mean absorbance of the released dye was 0.69 ± 0.33 (mean $\pm$ SD, $n = 30$). In all instances, bacteria appeared to have coated the base of the wells but, in some, the film of cells was continuous and uniform while, in others, the major deposition of cells was in a ring around the edge of the base.

Cell fusions and cloning

Of the 96×2 ml cultures prepared from the fusion with cell line Y3 a total of 42 showed confluent growth of hybrids after 14 d and 24 of these were screened as positive by ELISA. Ten cultures whose supernatants consistently gave high absorbance values on screening by ELISA were cloned in soft agar and the clones were developed for screening and production of McAb. The distribution of absorbance values obtained on screening duplicate supernatants from a single batch of 120 clones by ELISA is shown in Fig. 3. On the basis of such information, five clones strongly positive (++) for McAb production were selected for further investigation. These clones will be referred to as AFRC MAC 95 to 99.

Binding-competition assays

Competition between labelled and unlabelled McAb for binding to *Butyrvibrio* PI-7 cells indicated that the five McAb were of three types (Fig. 4). Each McAb strongly inhibited the binding of its ³H-labelled counterpart, but in all cases, dilution of unlabelled McAb between 20

Fig. 3. Screening of culture supernatants by ELISA. Clones obtained from confluent hybrid cultures were assayed for the presence of antibody after 7 to 10 d, as described in Methods. Background absorbance ranged from 0 to 0.15 and clones were scored weak +, + or ++, according to the absorbance obtained in the ELISA (1–2 times, 2–3 times or more than 3 times the background value, respectively).

Fig. 4. Classification of McAb by binding-competition assay. Experiments were done as described in Methods. Degree of competition (percentage inhibition of binding) equates with extent of shading as follows: ■, >50%; ▨, 25–50%; □, <25%.

and 50 times eliminated competition. The first type (type I) was represented by MAC 95 which competed only weakly with MAC 98 and MAC 99 for binding to *Butyrvibrio* PI-7, but inhibited binding of MAC 96 and MAC 97 by 25 to 50%. Type II McAb, MAC 96 and MAC 97, inhibited binding of MAC 98 and MAC 99 by 25 to 50% and competed strongly (>50% inhibition of binding) with the others. MAC 98 and MAC 99 (type III) strongly inhibited binding of all ³H-labelled McAb to *Butyrvibrio* PI-7 cells.

Cross-reaction of McAb with rumen genera other than *Butyrvibrio*

All five McAb failed to cross-react with a selection of type cultures comprising *Megasphaera elsdenii*, *Ruminococcus albus* (two strains), *Ruminococcus flavefaciens*, '*Butyribacter*' sp. (three strains; White, 1969), *Selenomonas ruminantium* (two strains), *Bacteroides ruminicola* subsp. *ruminicola* and subsp. *brevis*, *Streptococcus bovis* and '*Fusocillus*' sp. (two strains; Kemp *et al.*, 1975). A consistent but weak cross-reaction (<20% reaction obtained with *Butyrvibrio* PI-7) with *Bacteroides amylophilus* H18 was exhibited by MAC 95, but not by the remaining four McAb.

Fig. 5. Cross-reaction of presumptive butyrvibrios and type cultures with antiserum and McAb prepared against *Butyrvibrio* PI-7. Butyrvibrio cultures were screened by ELISA as described in Methods. In the absence of cross-reaction the A_{450} was <0.1 (□); positive cultures were scored ■ (A_{450} 0.1 to 0.5) or ■ (A_{450} >0.5).

Cross-reaction of rat antiserum and McAb with presumptive butyrvibrios

A total of 63 presumptive butyrvibrios were examined for cross-reaction with rat antiserum prepared against *Butyrvibrio* PI-7, using the ELISA. The butyrvibrios had been obtained primarily from cows (27 strains; Hazlewood *et al.*, 1983), from reindeer (20 strains; Orpin *et al.*, 1985) and from sheep (11 strains; Greenwood *et al.*, 1983), but included five well-characterized (type) strains donated by other workers, namely strains Bu49, Nor37 and Il6-31 (Sharpe *et al.*, 1975) strain LM8/IB (Hazlewood & Dawson, 1975) and strain B835 (Henderson, 1973). Of the 63 butyrvibrios, 21 gave a positive reaction with antiserum (Fig. 5). A strong reaction was observed between *Butyrvibrio* PI-7 and its homologous antiserum but five other bovine and one ovine isolate cross-reacted equally strongly. A weaker cross-reaction was evoked by 14 other isolates, including representatives of each of the sources listed (Fig. 5). Two of the type cultures, Bu49 and Nor37, did not cross-react with PI-7 antiserum.

Of the 63 butyrvibrios, 13, including *Butyrvibrio* PI-7, were able to bind one or more of the five McAb (Fig. 5). Depending on the extent of cross-reaction of each isolate with the panel of five McAb, it was possible to divide these strains broadly into two groups. The first group (Fig. 5) cross-reacted extensively and contained strain PI-7 and four other strains which, like PI-7, were strongly proteolytic isolates from forage-fed cows (Hazlewood *et al.*, 1983). The second group (Fig. 5) cross-reacted with MAC 95 or MAC 99 or both and contained six strains isolated from sheep and one strain isolated from a cow. The only type culture to bind McAb was Il6-31 and this organism cross-reacted with MAC 95 only.

Biochemical characteristics of presumptive butyrvibrios

The obligately anaerobic Gram-negative rods which had been isolated from cows and sheep were assigned to the genus *Butyrvibrio* on the basis of biochemical characterization as described in standard texts (Hungate, 1966; Holdeman & Moore, 1972; Ogimoto & Imai, 1981; Table 1), and by comparison with strains whose status as *Butyrvibrio* spp. had been deduced previously, in some instances, by other workers (Bu49, Nor37 and Il6-31, Sharpe *et al.*, 1975; LM8/IB, Hazlewood & Dawson, 1975; B835, Henderson, 1973). Table 1 lists the fermentation characteristics of these type cultures together with the characteristics of a selection of the presumptive butyrvibrios used in this study. All other isolates were tested similarly and, with the exception of minor differences in substrate fermentation patterns and some variation in the extent to which cells were curved, all isolates gave the same results and were assumed to be members of the genus *Butyrvibrio*.

DISCUSSION

The ELISA described in this paper is clearly a rapid, sensitive and economical way of investigating and quantifying the reaction of a bacterial surface antigen with antiserum or McAb.

The results for the dye-release assay support the conclusion that the binding efficiency of bacterial cells is comparable for different species of bacteria. Data obtained with the two butyrivibrios (strains PI-7 and Bu49) indicate that bacterial films formed from similar concentrations of cells of closely related bacteria take up equivalent amounts of dye but for unrelated bacteria the assay provides only a qualitative indication of binding. PAGE blue G90 binds mainly, but probably not exclusively, to protein and it is not unreasonable to suppose that organisms of one genus may contain similar amounts of dye-binding components per unit dry weight, while dissimilar organisms may not.

Fixed cells of *Butyrivibrio* PI-7 were not very immunogenic in rats; three booster doses were required after the initial immunization before antibodies were detected. Three of the five type cultures and 17 of the 63 presumptive butyrivibrios cross-reacted with antiserum raised against *Butyrivibrio* PI-7. This lack of specificity manifest by antiserum prepared against a butyrivibrio is consistent with the findings of others (Margherita & Hungate, 1963) and provides further evidence for the heterogeneous nature of this taxonomic group.

Five McAb raised against *Butyrivibrio* PI-7 were classified into three types on the basis of their competition for binding to cells of strain PI-7, indicating that there are at least three types of antigenic determinant on the surface of PI-7. Of the 20 butyrivibrios which cross-reacted with the antiserum, 12, including type culture Il6-31, also gave a positive response with one or more of the five McAb. These strains could be placed into two groups according to their pattern of cross-reaction (Fig. 5). Reaction with MAC 96 and MAC 97, or with all McAb, was limited to four bacteria which, like strain PI-7, were proteolytic butyrivibrios isolated from the rumen of the two cows sampled in an intermixing group of three. These four strains (PI-23, PI-25, PI-26 and PI-27) did not differ greatly from PI-7 in biochemical tests. Considering the different species of ruminant from which cross-reacting butyrivibrios were obtained and the extreme geographical separation between animals from which cross-reacting organisms were isolated, it would appear that MAC 95 and MAC 99 may be directed against more widely distributed and conserved antigenic determinants, while MAC 96, MAC 97 and MAC 98 are specified by strain-specific determinants which may be unique to proteolytic butyrivibrios such as strain PI-7. The fact that rat antiserum cross-reacted with a further eight butyrivibrios, including two other type bovine cultures and two other proteolytic bovine isolates, indicated that the panel of five McAb used here does not represent every one of the surface antigens on strain PI-7. Loss of antigenic determinants by *Butyrivibrio* type cultures or the possibility that these apparently similar organisms are not in fact members of the genus *Butyrivibrio* could be regarded as alternative explanations. The weak cross-reaction between *B. amylophilus* strain H18 and MAC 95 was an unexpected observation. With this exception it appears that *Butyrivibrio* PI-7 and the type cultures of the other genera examined do not share antigenic determinants.

It has been concluded that McAb can complement standard biochemical procedures routinely used in identifying methanogenic archaeobacteria, by specifying unique molecular markers for each taxonomic category (Jovell *et al.*, 1981; Conway de Macario *et al.*, 1981; Macario & Conway de Macario, 1982). Results described here suggest that McAb prepared against a rumen butyrivibrio are directed either against highly strain-specific determinants (MAC 96, 97 or 98) or against determinants more generally distributed and conserved within the genus (MAC 95 or 99). The pattern of cross-reaction of presumptive butyrivibrios with this panel of McAb can clearly be used to subdivide isolates which appear to be identical when subjected to standard biochemical procedures. Whether antigenic relatedness is a valid characteristic for establishing further taxonomic divisions in the genus *Butyrivibrio* will depend on further studies with larger numbers of McAb. However, a relatively small panel of McAb clearly has the specificity necessary for construction of a probe to follow the fate of a specific organism, such as a genetically engineered bacterium, introduced in the rumen. In this context, the most useful McAb will be those which recognize genetically stable determinants.

We would like to acknowledge Rebecca Morley and Sarah Hitching for skilled technical assistance, and Dr G. W. Butcher for helpful discussions.

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