Human portal serum stimulates cell proliferation in immature *Schistosoma mansoni*

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SUMMARY

Schistosomula of *Schistosoma mansoni* were incubated in RPMI 1640 medium containing 10% fetal calf serum, 10% human portal venous or 10% human peripheral venous sera in the presence of bromodeoxyuridine (BrdU) in order to measure differences in cell proliferation. The rates of cell proliferation as expressed by BrdU labelling indices (BLI) were determined as a function of time of incubation by immunohistochemistry using monoclonal antibody to BrdU. Compared to schistosomula cultured in the presence of RPMI plus 10% fetal calf serum, BLIs were increased by 60% in the presence of human portal, but not in peripheral serum. This stimulatory effect was substantially reproduced by a fraction of portal serum with a molecular weight range between 1 and 50 kDa. However, in the presence of human peripheral venous serum, either whole or fractionated, schistosomula showed no significant difference compared to RPMI plus 10% fetal calf serum alone. Furthermore, human portal serum fractions of molecular weight greater than 50 kDa also revealed no significant difference relative to control. The results indicate that portal venous serum component(s) of a molecular weight range higher than most simple nutrients can greatly stimulate the rate of cell proliferation of *Schistosoma mansoni* schistosomula.

Key words: *Schistosoma mansoni*, serum fractions, cell proliferation, BrdU.

INTRODUCTION

In susceptible hosts, the various species of *Schistosoma* bear a remarkable predilection for particular anatomical sites (Cheever, 1965), resulting in complications in specific organ systems. In man, the preference of *Schistosoma haematobium* for the veins of the urinary system causes inflammation and fibrosis of the bladder and/or ureters (Warren, 1978*a*). Similarly, localization of *Schistosoma mansoni* and *Schistosoma japonicum* in the human portal–mesenteric venous system results in colonic and hepatic granulomatous disease with progressive hepatic fibrosis that manifests itself in the morbid complications of portal hypertension (Warren, 1978*b*). From a teleological perspective, it seems that the localization phenomenon represents an advantageous evolutionary adaptation in that it simplifies the task of locating mates by bringing both sexes together in a relatively confined vascular region. In addition, deposition of eggs in these areas permits their elimination from the host for completion of the parasitic life-cycle. The mechanism by which the parasite determines this site preference, however, is unknown.

In considering this question, we noted the elegant series of studies by Miller & Wilson (1978, 1980), Wilson *et al*. (1978) and Knopf, Mangold & Makari (1983), who demonstrated that schistosomula of *S. mansoni* migrate through the venous and arterial vascular systems prior to their arrival in the portal-mesenteric system. In fact, it was determined that immature parasites make several passages through the pulmonary–systemic circulation before chance migration to the preferred site (Miller & Wilson, 1978). This occurs regardless of the site or mode of entry e.g. percutaneous, subcutaneous, intrapleural, or intracardiac. When schistosomula were injected directly into the portal vein, the majority of the parasites remained at that site (Miller *et al*. 1978). These data indicate that as a consequence of their migrations, the immature organisms are exposed to various regions of the circulatory system, and yet they exhibit a definite preference for the portal–mesenteric venous system.

The work of Taylor, Amin & Nelson (1969), and Taylor (1971) also showed that in single-sex infections, schistosomes migrated to the portal–mesenteric venous system, indicating that each sex is capable of locating the preferred site independent of the other sex. Blood draining to the portal vein is

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derived from the gastrointestinal tract. Therefore, it is different from peripheral blood in many respects (Ishikawa, 1976). We wondered whether the site preference of *S. mansoni* could be dependent on a constituent of portal blood that is not present in the periphery. This might take the form of a substance that the parasite recognizes or requires to develop.

In this regard Wu *et al*. (1985) showed that portal serum and a fraction ranging from 2 to 50 kDa stimulated egg deposition of *S. mansoni* worms *in vitro*. The range of these low molecular weight substances was larger than would be expected for simple carbohydrates, amino acids or free fatty acids absorbed from the gastrointestinal tract.

We hypothesized that a factor in portal venous blood could signal or be responsible for rapid growth of the parasite and preclude further migration based on the size of the organism. To test this hypothesis, the effects of portal and peripheral venous blood samples on cell proliferation of the organism were assessed. This could be accomplished by evaluating bromodeoxyuridine (BrdU) incorporation into cells. BrdU, which is a nucleoside analogue of thymidine, has been shown to be incorporated into DNA in place of thymidine in the course of S-phase of cell cycles (Mourad *et al*. 1993).

Therefore, the presence BrdU in nuclei could be used as a measure of the number of proliferating *S. mansoni* cells incubated under various conditions followed by immunohistochemical staining with anti-BrdU. To test the hypothesis, immature rather than adult *S. mansoni* were selected to simulate the situation shortly after arrival of these organisms in the portal venous circulation.

MATERIALS AND METHODS

Preparation of human portal and peripheral venous serum

In collaboration with Dr Refaat Kamel (Ain Shams University Hospital, Cairo, Egypt), following pre-operative fasting, human portal venous blood was obtained with informed consent from an adult Egyptian male with post-hepatitic cirrhosis for which he underwent a portacaval shunt operation. During the operation, portal pressures are routinely measured by direct puncture to determine the adequacy of the procedure, and portal blood was withdrawn through the catheter. The patient received isoflurane, Deprivan, nitrous oxide and oxygen during the procedure, and vitamin K, Dicynone, glucose 25 %, and Ringer's and glucose solutions during the post-operative period. Peripheral blood was drawn from the antecubital vein of the same patient. The blood was allowed to clot and spun down at 850 ***g*** to obtain the serum. Sera obtained in this manner were either used immediately or within 2 weeks of collection after storage at −70 °C.

Fractionation of human serum by ultrafiltration

The human serum samples 1·5 ml each were first filtered using a pressure dialysis chamber and filtration membranes, XM50 (Amicon Corp., Danvers, MA, USA) with an exclusion limit of 50 kDa. The filtration was performed using 150 ml of PBS buffer under conditions recommended by the company. The fraction retained by the filter (greater than 50 kDa), was washed with another 150 ml of PBS buffer, and the filtrate (300 ml) was dialysed through membranes with an exclusion limit of 1 kDa. All the serum fractions were concentrated to the original volume of 1·5 ml using these membranes.

Assay for in vitro *cell proliferation*

Schistosomula were collected by sterile perfusion (Duvall & DeWitt, 1967) from the livers of golden hamsters infested with 500–1000 *S. mansoni* cercariae 20 days after injection. Twenty to 30 schistosomula per dish were incubated for 1–5 days under 5 % CO_2 at 37 °C in (1) RPMI 1640 medium with penicillin (100 units/ml) and streptomycin (0·1 mg/ml) plus 10 % fetal calf serum (FCS); (2) the above RPMI/FCS medium plus 100 mmole BrdU; (3) the above RPMI/FCS medium plus 100 mmole BrdU and 10 % whole portal human serum; (4) RPMI/FCS medium plus 100 mmole BrdU and 10 % of the > 50 kDa human portal serum fraction; (5) RPMI/FCS medium plus 100 mmole BrdU and 10 % of the fraction ranging from 1 to 50 kDa of human portal serum. Analogous samples were prepared with human peripheral serum and its fractions.

After incubation, samples were fixed in 70 % ethanol in glycine buffer, pH 2, and then embedded in paraffin blocks. Sections of 5 μm thickness were cut and stained immunohistochemically with anti-BrdU using a kit as described by the manufacturer (Boehringer Mannheim Co.) to measure the number of proliferating cells. Adjacent sections of the same worm were stained identically with haematoxylin to visualize and allow counting of total cells present in the section. The BrdU labelling indices (BLI) were determined according to the formula:

$$\text{BLI } (\%) = \frac{(\text{Number of nuclei stained by anti-BrdU} \times 100)}{(\text{Total number of nuclei stained by haematoxylin})}.$$

The results are expressed as means of ±S.E.M. in percentage units. The minimum number of worms in the samples was 6.

Results were analysed using a 1-way analysis of variance and Bonferroni *post-hoc* comparisons to assess the presence of significant differences between the groups.

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Table 1. Average bromodeoxyuridine labelling indices (BLI) in 20-day-old *Schistosoma mansoni* cultured for 5 days in media containing human serum components

Additions to RPMI medium + fetal calf serum	None	+ Whole portal serum	+ Whole peripheral serum	+ Portal > 50 kDa fraction	+ Peripheral > 50 kDa fraction	+ Portal 1–50 kDa fraction	+ Peripheral 1–50 kDa fraction
Range	11·5–31·8	25·6–38·5	14·5–29·6	22·1–28·8	14·3–28·4	22·0–41·1	13·5–26·2
Means ± S.E.M.	20·9 ± 1·6	33·5 ± 1·5	21·7 ± 1·2	24·0 ± 0·97	21·6 ± 1·6	28·1 ± 2·9	21·0 ± 1·7
P values*	< 0·05	—	< 0·05	< 0·05	< 0·05	—	< 0·05

* Statistical significance when compared to RPMI/fetal calf serum plus whole human portal serum.

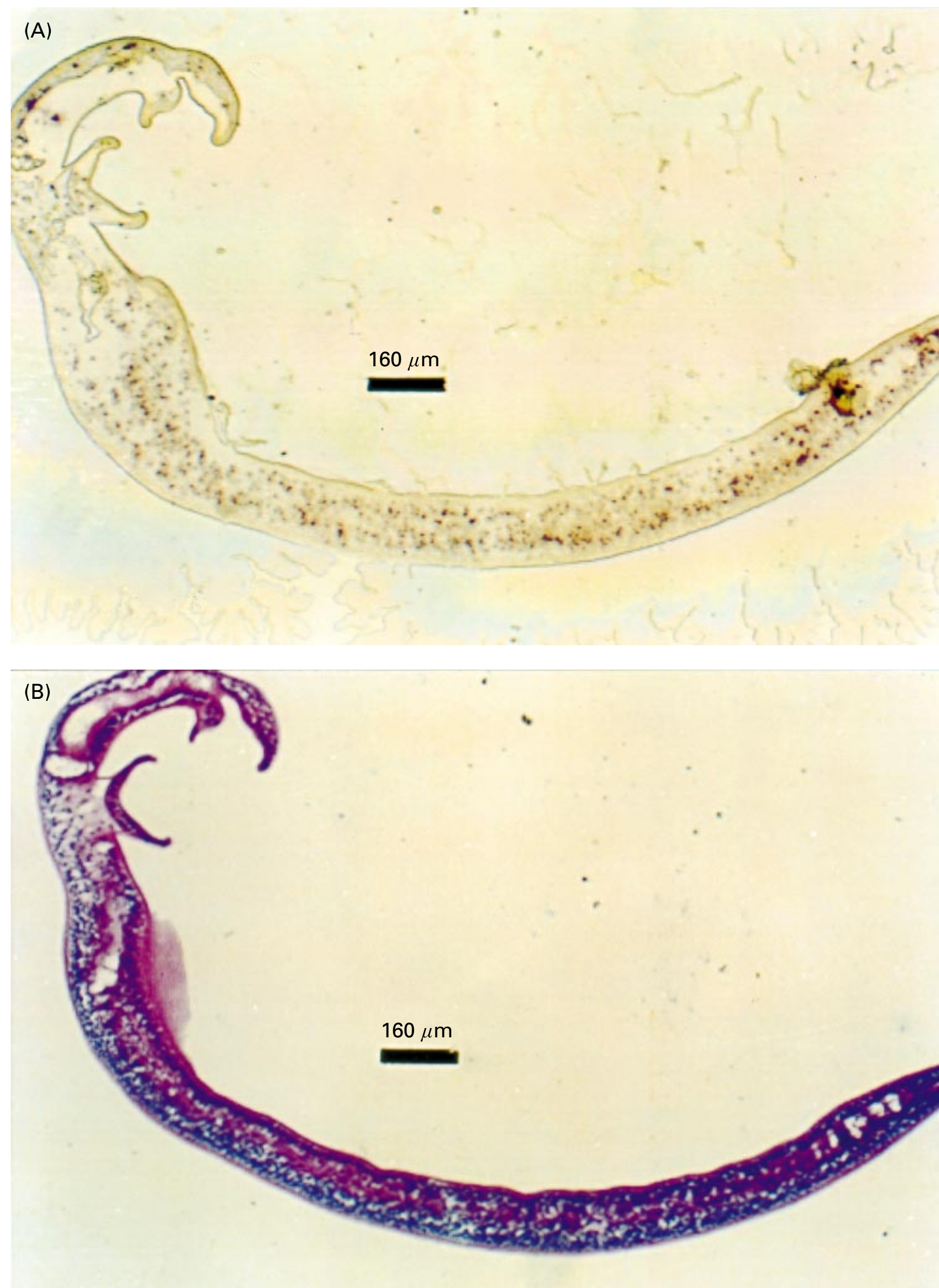

Fig. 1. (A) A representative example of a 23-day-old *Schistosoma mansoni* schistosomulum cultured in RPMI 1640 medium plus 10 % fetal calf serum and 10 % human portal serum for 24 h, labelled with BrdU and stained with anti-BrdU to reveal proliferating cells as described in the Materials and Methods section. (B) The same organism as shown in (A) stained with haematoxylin showing total nuclei. The BLI was calculated to be 29·6 %.

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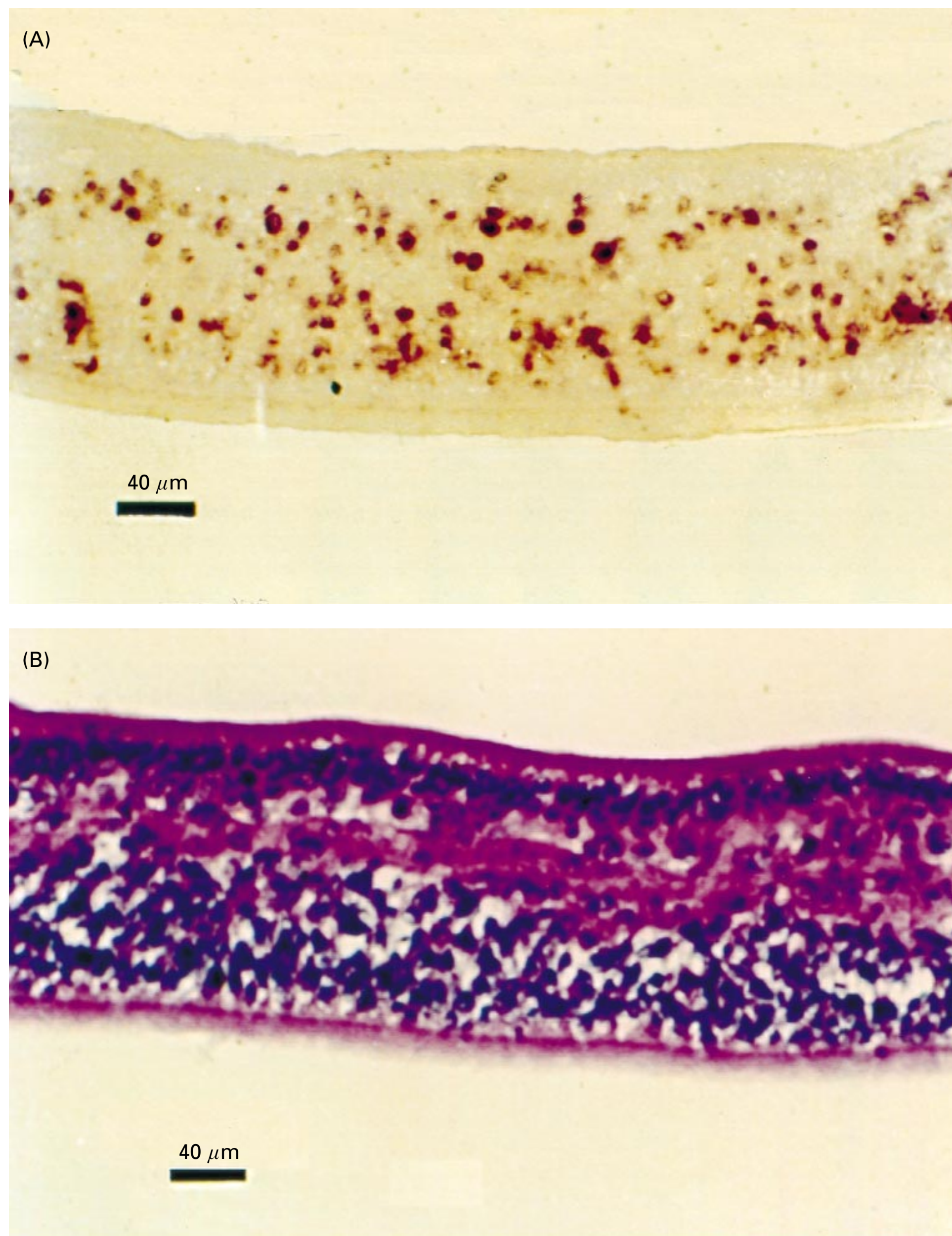

Fig. 2. (A) A representative example of the same 23-day-old *Schistosoma mansoni* shown in Fig. 1 (A) but at 400 × magnification to show more clearly the nuclear staining. (B) The same organism as shown in (A) stained with haematoxylin showing total nuclei.

RESULTS

Table 1 shows the mean values of BLI for 20-day-old schistosomula cultured for 5 days in media containing various serum fractions. The mean BLI of schistosomula in control medium (RPMI plus 10 % fetal calf serum) alone over this period was 20·9 %. However, in whole human portal serum, the BLI of 33·5 % was substantially and significantly greater under identical conditions. That this was not simply due to the human versus bovine source of the serum was shown by the results of incubation in the whole human peripheral serum column where the BLI was only 21·7 %. This was not significantly different from control medium, RPMI/FCS. Of interest was the fact that schistosomula cultured in portal serum fractions of 1–50 kDa had almost as high a BLI as whole portal serum, 28·1 %, while the corresponding fraction of peripheral serum had a BLI of only 21·0 %. The former was, but the latter was not, significantly different from control RPMI/FCS. Further evidence that the stimulation of proliferation was not due to a non-specific effect of human serum is shown by the BLI values following incubations in portal > 50 kDa fractions, 24·0 % and peripheral 21·6 %, respectively which were both *not* significantly different from control.

Fig. 1 A shows a representative 23-day-old schistosomulum, cut and mounted in longitudinal section after culture and labelling in RPMI/FCS medium

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Table 2. Average bromodeoxyuridine labelling indices (BLI) to 20 to 25-day-old *Schistosoma mansoni* cultured for 24 h in media containing human serum components

Additions to RPMI medium + fetal calf serum	None	+ Whole portal serum	+ Whole peripheral serum	+ Portal > 50 kDa fraction	+ Peripheral > 50 kDa fraction	+ Portal 1–50 kDa fraction	+ Peripheral 1–50 kDa fraction	+ Whole portal and peripheral
Range	12·2–20·5	22·2–37·7	13·3–22·2	13·2–17·5	10·4–21·5	20·4–33·3	12·4–23·0	19·8–38·7
Means ± S.E.M.	16·7 ± 1·2	27·4 ± 1·8*	18·6 ± 1·2	15·7 ± 0·6	16·4 ± 1·1	27·9 ± 1·5*	17·7 ± 3·5	29·3 ± 1·8

* $P < 0{\cdot}05$ compared to RPMI/fetal calf serum alone.

Table 3. Multiple direct comparisons: differences in average BLI of 20 to 25-day-old *Schistosoma mansoni* schistosomules cultured for 24 h in RPMI/FCS media containing human serum components

(A, RPMI/FCS; B, whole peripheral; C, whole portal; D, portal and peripheral; E, portal > 50 kDa; F, peripheral > 50 kDa; G, portal 1–50 kDa; H, peripheral 1–50 kDa.)

	A	B	C	D	E	F	G
B	1·87						
C	10·7*	8·84*					
D	12·6*	10·8*	1·91				
E	−0·97	−2·82	−11·7*	−13·6*			
F	−0·34	−2·20	−11·0*	−13·0*	0·61		
G	11·2*	9·31*	0·48	−1·44	12·1*	11·5*	
H	0·96	−0·90	−9·74*	−11·7*	1·92	1·30	−10·2*

* $P < 0{\cdot}05$.

containing 10% portal serum for 24 h. All organisms maintained motility throughout the culture period. The labelled cells were most dense in the gut, but were also present throughout the body. Fig. 1B shows the same organism as in Fig. 1A stained with haematoxylin to visualize total nuclei. The organism is completely intact and the staining is uniform. The labelling index was calculated to be 29·6%. Fig. 2A shows the same organism as in Fig. 1A, but at higher power (400×) to better appreciate the nuclear staining. Fig. 2B shows a haematoxylin stain of the same organism as in Fig. 2A.

Culture and labelling of a 23-day-old schistosomulum in RPMI/FCS medium containing 10% peripheral serum for 24 h resulted in a labelling index calculated to be 21·7%. Finally, incubation of a 23-day-old schistosomulum in RPMI/FCS alone after anti-BrdU staining was qualitatively similar to that after culture in human peripheral serum, and quantitatively similar as well with a calculated BLI of 20·5%. The total number of nuclei counted for the 23-day-old worm in RPMI alone was 922, in peripheral serum 778, and portal serum 1322.

To determine whether a shorter term incubation would make a difference, equal numbers of *S. mansoni* aged 20–25 days were cultured in various media for 24 h, then fixed in 70% ethanol. The labelled cell counts of all organisms were calculated as means ± S.E.M., and the results are shown in Table 2. The BLI of organisms cultured in whole peripheral serum, peripheral serum 1–50 kDa, and > 50 kDa fractions were 11·4% higher, 6·0% higher and 1·7% lower, respectively than control RPMI/FCS. None of these values were significantly different compared to control. Similarly, in portal serum fractions > 50 kDa, the BLI was 5·9% lower, but did not differ significantly from control. As seen in the 20-day-old organisms, the portal 1–50 kDa fraction approached the level of stimulation of whole portal serum. That these results were not due to some growth suppressive effect of peripheral serum is shown in the last column of Table 2 where whole peripheral serum was added to whole portal serum, to make a final concentration of 10% for each. This mixture performed as well as whole portal serum alone, indicating that peripheral serum does not have an inhibitory factor that could account for the observed differences in proliferation.

While comparisons to RPMI/FCS controls were informative, direct comparisons between media are more definitive. Table 3 shows such direct comparisons of BLI for organisms grown in various media for 24 h. The labelling indices of schistosomula incubated in media containing whole portal

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serum were significantly different from media containing *all* non-portal serum components and also the > 50 kDa portal fraction. Similarly, whole peripheral serum effects were significantly different from *all* media containing portal serum except the > 50 kDa fraction. The effects of whole portal serum on BLI were not significantly different from those of the 1–50 kDa fraction.

DISCUSSION

We have shown previously that medium containing portal serum from non-human hosts increased the rate of egg production from schistosomes. This phenomenon was observed in the presence of portal serum from susceptible and non-susceptible laboratory animal hosts, but not in peripheral serum from any of the animals studied. Furthermore, a 2–50 kDa portal serum fraction was the most effective component in stimulating oviposition (Wu *et al*. 1985). It was concluded that a stimulatory substance or substances was present in portal serum that was absent in peripheral serum or in blood regardless of the susceptibility of animals to *S. mansoni* infestation (Wu *et al*. 1986). The actual factors and mechanism responsible have not yet been identified.

The present studies confirm that not only laboratory animals, but indeed, *human* portal serum can be discriminated from peripheral serum by developing schistosomula. The consistently higher labelling indices in organisms cultured in medium containing portal serum compared to peripheral serum suggest that factor(s) involved in proliferation may be responsible for the previous observations.

The effects of portal versus peripheral serum could have been explained by a growth inhibitory factor present in peripheral serum that was absent in human portal and fetal calf serum. However, the mixing experiment in which portal serum was combined with peripheral serum showed that this mixture was as effective as whole portal serum itself, arguing against the presence of an inhibitory substance in peripheral serum.

An obvious difference between portal serum and serum from the rest of the body is that the former contains constituents absorbed from the gut. Nutrients from digestion as well as bacterial products can be present. Such a difference would be most marked in a post-prandial state. However, the patient from whom this serum was obtained was obliged to have nothing by mouth for at least 18 h due to pre-operative requirements. To further characterize the components of portal serum involved, sera were dialysed to remove low molecular weight substances. This filtration of serum constituents of molecular weights < 1 kDa did not decrease the effectiveness of portal serum on growth. Clearly, the filtration limits of the dialysis tubing are nominal, and the actual materials that are retained by the membranes depend on their shape and configuration. It is possible that low molecular weight substances that adhere or bind to larger serum proteins such as albumin, transferrin and others in portal serum could be responsible for the observed results. Nevertheless, the direct comparisons of the effects of various serum components on short incubations indicate that a substantial proportion of the growth stimulatory activity in whole portal serum can be attributed to the 1–50 kDa fraction, and the observed proliferative effect of whole portal serum is not likely due to the presence of simple nutrients such as mono- and disaccharides, amino acids, and low molecular weight fatty acids.

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