Exchange of Macromolecules between Plasma and Peritoneal Cavity in Ascites Tumor-bearing, Normal, and Serotonin-injected Mice

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ABSTRACT

Fluorescein-labeled dextrans (FITC-D) from 3 to 5000 kDa (Stokes' radii from 1 to 40 nm) were used to study influx from the plasma into the peritoneum and efflux from the peritoneal cavity into the plasma in normal and ascites tumor-bearing mice and in mice whose peritoneal vessels had been rendered hyperpermeable by serotonin. Two syngeneic transplantable murine ascites tumors were studied: mouse ovarian tumor and the TA3/St breast adenocarcinoma. To control for effects of peritoneal fluid volume, influx and efflux were also analyzed in mice that had received 5 ml of 5% bovine serum albumin i.p. as "artificial ascites." Following i.v. or i.p. injection, levels of FITC-D in the plasma and peritoneal fluid were quantitated by fluorimetry at successive time intervals from 5 to 360 min posttracer injection. Influx and efflux data were analyzed with a model consisting of three compartments (plasma, peritoneal cavity, and the extravascular space of all other organs) to yield kinetic parameters that characterize macromolecular transport. The dependence of the FITC-D tracer, from 3- to 50-fold more FITC-D accumulated in mouse ovarian tumor or TA3/St tumor ascites fluid, and 3- to 10-fold more FITC-D accumulated in the peritoneum of serotonin-treated than normal mice, all of it intact by gel exclusion chromatography. Influx of the FITC-D from plasma into the peritoneum, as characterized by the rate constant $k_i$, was 2- to 40-fold greater in ascites tumor-bearing animals and 2- to 10-fold greater in serotonin-treated animals than in controls. Control animals with artificial ascites showed at most a 4-fold increase in the value of $k_i$. As judged by fluorescence microscopy, the permeability of peritoneal-lining vessels in ascites tumor-bearing animals was greatly increased to FITC-D of 70 to 5000 kDa. Efflux of FITC-D, characterized by the rate constant $k_o$, was reduced from 5- to 50-fold in ascites tumor-bearing animals but was unchanged or actually somewhat enhanced following serotonin treatment. Efflux in animals that had received artificial ascites was reduced 2.5- to 12.5-fold, correlating increased peritoneal fluid volume with decreased efflux. We conclude that tracer accumulation in malignant ascites fluid results from both increased influx as well as impairs efflux. Influx, and to a lesser extent efflux, were significantly affected by tracer size. However, within the range of FITC-D tested, we found no absolute size barrier to macromolecular transport from plasma to the peritoneal cavity, or vice versa. Our results may aid in the design of therapeutic macromolecules whose properties favor their escape from tumor vessels and their accumulation in tumor ascites.

INTRODUCTION

The microvasculature of many autochthonous and transplantable solid tumors is hyperpermeable to plasma proteins and other circulating macromolecules (1-8). In several transplantable solid carcinomas, leaky vessels represent a discrete subpopulation of vessels that is comprised of well-differentiated venules and small veins; these are found in greatest concentration at the tumor-host interface (9). Of interest, this distribution of leaky vessels corresponds closely to that of extravascular fibrin deposits that are regularly found in the same tumors (1, 2, 6, 7). Fibrin deposits result from extravascular clotting and cross-linking of plasma fibrinogen that has extravasated from leaky tumor blood vessels. These extravascular fibrin deposits undergo extensive local turnover and provide a provisional matrix for the generation of mature tumor stroma (10-13).

Abnormal fluid accumulation in the form of a plasma protein-rich exudate also accompanies the ascites form of tumorigrowth that occurs in serous cavities (14-19). However, the generation of tumor ascites has not been carefully elucidated. By analogy with solid tumors, it has been suggested that ascites fluid accumulation results from hyperpermeability of the blood vessels that line serous cavities (14-19). Consistent with this view, plasma proteins of the size of albumin (69 kDa) have been shown to enter tumor ascites fluid more rapidly than the peritoneal cavities of normal mice (14-19). Alternatively, it has been argued that ascites fluid accumulation results from impediment of fluid drainage, as from obstruction of draining lymphatics by invading tumor cells (20, 21). Once present, ascites fluid may be expected to exert an effect on both peritoneal influx and efflux (22). Whatever its pathogenesis, the protein-rich exudate that comprises tumor ascites differs from that found in solid tumors in that it remains fluid and generally is not transformed into an insoluble fibrin gel (10, 12, 23, 24), despite the fact that tumor ascites is rich in procoagulants (25).

One possible explanation for the absence of fibrin clot in ascites tumors is that the permeability of vessels lining serous cavities is more restrictive than that of vessels supplying solid tumors; according to this view, peritoneal vessels allow the free passage of smaller plasma proteins such as albumin but retain larger molecules such as fibrinogen (340 kDa). Consistent with this hypothesis, ascites fluids are reportedly depleted of fibrinogen, as compared with several smaller plasma proteins (27).

The present experiments were undertaken to investigate the influx and efflux of macromolecules into and out of the peritoneal cavities of ascites tumor-bearing mice. Previous studies of peritoneal transport have focused primarily on the normal peritoneum and on solutes of <5 kDa (28), although a few reports have also analyzed transport of macromolecules from 20 to >700 kDa in normal animals (29-32). However, to our knowledge, peritoneal transport of large macromolecules has not been measured in ascites tumor-bearing animals.

Fluorescein-labeled dextrans ranging from 3 to 5000 kDa were used as tracers. FITC-D4 have the advantage over plasma protein tracers in that they are available in a wide range of sizes, are metabolized slowly in vivo, and are not readily trans-
formed or degraded by plasma or tissue enzymes likely to be activated at sites of plasma extravasation (33). We compared tracer influx and efflux in ascites tumor-bearing animals with those of normal mice and with mice whose peritoneal blood vessels were rendered hyperpermeable by serotonin. We also analyzed the effect of peritoneal fluid volume on macromolecular transport in animals that received 5 ml of 5% BSA i.p. as "artificial ascites." We then fit our data to a three-compartment model (34) so that transport rates of tracers between the plasma, the peritoneal cavity, and other tissues could be expressed quantitatively.

MATERIALS AND METHODS

Experimental Design. Influx and efflux of macromolecules into and out of the peritoneal cavity were studied and compared in four treatment groups: ascites tumor-bearing mice, normal controls, mice given injections i.p. of serotonin (10 μg in 0.5 ml of saline), a permeability-enhancing amine, and mice given injections i.p. of 5 ml of 5% BSA in saline. In some experiments, BSA was injected in HBSS instead of saline; in other experiments, 5% endotoxin-free human serum albumin was injected instead of BSA. Young adult (6- to 8-week-old) mice received 1 x 106 tumor cells i.p. Seven days (TA3/St) or 10 days (MOT) later, animals were given injections i.v. of 0.2 ml of fluorescein-labeled dextran tracers (FITC-D) (30 mg/ml), or i.p. of 1 ml of FITC-D (3 mg/ml). At various intervals thereafter, animals were anesthetized with ether and blood samples were collected by retroorbital puncture into a known volume of heparin. Immediately thereafter, animals were sacrificed and exsanguinated. HBSS without phenol red indicator (2 ml) was injected i.p. and the contents of the peritoneal cavity were mixed. The peritoneal cavities were opened by a small ventral surgical incision and the peritoneal contents were recovered to the fullest extent possible, the total volume was recorded, and tumor cells were counted. The cell suspension was centrifuged (160 x g, 10 min, room temperature) and the cell-free supernatant was removed and its volume recorded. The error in volume recovered was found to be ≤3% in normal animals and was presumably lower in ascites tumor-bearing animals where peritoneal fluid volumes were larger. Platelet-poor plasma was prepared by centrifugation (15,600 x g, 10 min, 4°C) of heparinized blood. Aliquots of the plasma and cell-free peritoneal fluid were analyzed for FITC-D content by fluorometry at pH 7.4.

Tracers. Fluorescein-labeled dextrans with weight-average molecular weights of 70,000 and 150,000 were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. FITC-D of Mw 3,000 and 20,000 were from Sigma Chemical Co., St. Louis, MO. The average degree of substitution was ~0.001; i.e., one FITC group per 1000 glucose units. The ratio of the weight-average molecular weight to the number-average molecular weight of all of the FITC-D was <1.25, indicating a narrow polydispersity. Unlabeled dextran of Mw 5,000,000 (Sigma) was covalently labeled with fluorescein isothiocyanate (Sigma) (35) with a resulting degree of substitution of 0.004. Both commercial FITC-D and the FITC-D we synthesized were free of detectable free fluorescein as judged by gel exclusion chromatography and ethanol precipitation. Solutions of FITC-D for injection were prepared in 0.9% NaCl solution. Dextran concentrations were determined by the anthrone reaction (36). Stokes' radii for the various FITC-D are summarized in Table 1 (37). Serotonin (5-hydroxytryptamine, creatinine sulfate complex) and BSA were purchased from Sigma, and HBSS was purchased from Gibco, Grand Island, NY. Five % endotoxin-free human serum albumin was obtained from Alpha Therapeutics Corp., Los Angeles, CA.

Animals and Tumors. Two transplantable murine ascites tumors were studied: MOT and the TA3/St breast adenocarcinoma, the kind gifts of Drs. Gerald Kolody and Barbara Sanford, respectively. Tumor cells (1 x 106) were passaged weekly in the peritoneal cavities of syngeneic C3HeB/FeJ and A/Jax mice, respectively. At the time of tumor harvest TA3/St and MOT tumors generated 2–3 ml and 8–10 ml of ascites fluid, respectively. Control A/J mice weighed 15–16 g and control C3HeB/FeJ mice weighed 20–25 g. At the time of harvest, TA3/St and MOT tumors generated 2–3 ml and 8–10 ml of ascites fluid, respectively. Control A/J mice weighed 15–16 g and control C3HeB/FeJ mice weighed ~22 g, and MOT tumor animals weighed ~30 g.

Preparation of Tissue for Fluorescence Microscopy. Immediately after removal of the peritoneal fluid, the entire peritoneal wall as well as the diaphragm and mesentery were rapidly excised and immersed in a 70:30 mixture of ethanol:10% formalin (38). Fixation proceeded for 4 h at room temperature. Subsequently, tissues were dehydrated in ascending grades of ethanol (70 to 100%) over 24 h and were cleared in xylene. Full thickness segments of the parietal peritoneal wall (up to 1 x 1 cm), diaphragm, and mesentry were mounted directly on glass slides and were coverslipped with immersion oil for fluorescence microscopy.

Gel Filtration. Aliquots of FITC-D and plasma and peritoneal fluids from animals given injections of FITC-D i.v. were fractionated by gel filtration chromatography on Sepharose CL-4B (Pharmacia) columns (1.5 x 35 cm) at 25°C in 0.3 m NaCl at an elution rate of 1 ml/min. Samples (0.2 ml) that contained approximately 5 μg (peritoneal fluid) or 50 μg (plasma) of FITC-D were applied to columns and 1-ml fractions were collected and analyzed by fluorometry.

Mathematical Model. Influx into the peritoneum requires that circulating molecules pass through a succession of barriers that include the vessel wall and its underlying basal lamina, interstitial connective tissue, and the mesothelium. Efflux from the peritoneal cavity into the blood requires that molecules either drain via lymphatic channels concentrated in the diaphragm or reenter the blood directly by retracing the steps summarized above for influx. Passage of macromolecules through the various vascular and peritoneal barriers may depend on either or both convection and diffusion. Influx and efflux are complex, multifaceted processes, depending at once on properties of the tracer molecule itself (e.g., size, shape, charge, interaction with plasma proteins, relative concentrations in the plasma, interstitial space and peritoneal cavity, etc.) and on host factors such as vascular permeability, hydraulic conductivity and reflectivity, the surface area available for intercompartmental exchange, rates of local blood flow, peritoneal fluid volume and composition, pressure gradients, etc. Additional factors may also influence molecular drainage via diaphragmatic lymphatics; e.g., rate and type of breathing, plugging of lymphatics by tumor cells, etc.

While a complete understanding of molecular transfer will require detailed control and measurement of each of these parameters (and perhaps others not yet recognized), it is useful, as a first approximation, to consider influx and efflux as composite entities; i.e., the net flux of tracers from the plasma into the peritoneum and from the peritoneum into the blood as represented by the summation of all of the variables listed above. For this purpose we chose a simplified mathematical model (34) composed of three compartments: the intravascular space (plasma), the peritoneal cavity (peritoneal fluid), and all extravascular spaces other than the peritoneal cavity (Fig. 1). This model makes no attempt to measure any specific physiological process. Rather, all factors that modify transport between compartments are lumped to factors that modify transport between compartments are lumped to additional factors that modify transport between compartments are lumped to...
vascular leakage. Extravasation of 5000 kDa FITC-D was evident microscopically within 15 min of tracer injection, initially taking the form of discrete, brightly fluorescent foci about individual small vessels (Fig. 2C).

In serotonin-injected animals, leakage of 70 kDa FITC-D tracer was detectable within 1 min after i.v. injection of tracer and became extensive by 15 min (Fig. 2D). As in tumor-bearing mice, sites of leakage were more precisely defined with the 5000 kDa FITC-D tracer, and were again associated with vessels having the appearance of venules and small veins (Fig. 2E).

In contrast to the 70 and 5000 kDa tracers, the 3 kDa FITC-D extravasated extensively from vessels in a wide variety of normal tissues including the peritoneal walls of both normal and ascites tumor-bearing animals (not illustrated).

Quantitation of FITC-D Influx by Fluorimetry. The concentration of FITC-D tracers of various sizes in the plasma and peritoneal cavity was measured at successive intervals after a single i.v. injection in four groups of mice: ascites tumor-bearing, serotonin-injected, normal, and normal with artificial ascites (Fig. 3). Like plasma proteins of similar size, FITC-D of ≥70 kDa was cleared slowly from the plasma of normal mice (plasma half-lives, >240 min); in contrast, the 3 and 20 kDa FITC-D tracers were cleared much more rapidly with measured plasma half-lives of <5 min and ~15 min, respectively. These smaller tracers are filtered and not resorbed by the kidney; also, the 3 kDa FITC-D extravasates from normal blood vessels into a variety of other tissues and subsequently may reenter the plasma directly or by way of lymphatics.

FITC-D of all five sizes entered the peritoneal cavities of normal mice at low but measurable rates (3 kDa > larger FITC-D) (Fig. 3). Maximum accumulation of the 3 and 20 kDa FITC-D, expressed as fraction (or percentage) of injected dose, occurred earlier (30 to 60 min) than for the larger tracers. However, in normal animals maximum measured accumulation differed little among FITC-D of widely varying size, in all cases being 0.1-0.4% of the injected dose. In fact, peritoneal accumulation was somewhat greater for the larger three tracers than for 20 kDa FITC-D, probably because the latter, like 3 kDa FITC-D, was cleared from the kidney and/or was more widely distributed in other normal tissues (Fig. 3).

Peritoneal accumulation of all five FITC-D was much greater in ascites tumor-bearing animals than in normal controls (Fig. 3). In ascites tumor animals, maximum peritoneal accumulation amounted to 1.0 to 11% of the injected dose, i.e., 3 to 50 times that of normal animals. Accumulation was similar for all tracers except the largest (5000 kDa) whose maximum was only 3- to 6-fold above normal, a significantly smaller increment than for the other tracers. In ascites tumor-bearing as in normal animals, the two smaller tracers achieved maximum peritoneal concentrations at earlier intervals (i.e., 30 min) after i.v. injection and thereafter plateaued or declined somewhat. In contrast, the larger tracers continued to accumulate gradually in ascites fluid for at least 3 to 4 h after i.v. injection (Fig. 3).

Tracer influx was also studied in normal animals that received a single i.p. injection of serotonin. In such animals, 1 to 3% of injected FITC-D accumulated in the peritoneal cavity by 30–60 min. This represents a 3- to 12-fold increase over that in normal control animals.

Finally, tracer influx was also studied in normal animals with artificial ascites (injection of 5 ml of 5% BSA i.p. along with the i.v. injection of tracer). In such animals, 0.4 to 4% of injected FITC-D accumulated in the peritoneal cavity by 30–60 min depending on the molecular weight of the FITC-D. This represents up to a ~4-fold increase in maximum accumulation over that in normal control animals.

Size Distribution of FITC-D in Plasma and in Peritoneal Fluid. Fractionation on Sepharose CL-4B of 5000 kDa FITC-D, and
Cavities of ascites tumor-bearing animals, comparable to the control animals (Fig. 5). More than 95% of tracers >20 kD compartment, a 10- to 50-fold reduction as compared with levels found in normal animals. Fraction of the 3 kDa FITC-D tracer escaped from the peritoneal bearing animals for at least 6 h (Fig. 5). However, a larger remained within the peritoneal cavities of the ascites tumor-bearing animals. Even at 6 h after i.p. injection, only 1% plasma levels of 3 kDa FITC-D fell, whereas, for the other tracers rose significantly in normal mice for at least 60 min cleared from the peritoneal cavity over the 6 h following i.p. control animals, FITC-D of differing size were progressively cleared from the peritoneal cavity the over the 6 h following i.p. injection (Fig. 5). Peritoneal clearance approximated a linear function on semilog plots and was affected to only a small extent by tracer size. Concomitant with peritoneal clearance, plasma levels of tracers rose significantly in normal mice for at least 60 min (Fig. 5). At 60 min, plasma levels of the 3 and 20 kDa FITC-D approximated 5% of the injected dose, and considerably more (10–50%) in the case of the larger tracers. Thereafter, plasma levels of 3 kDa FITC-D fell, whereas, for the other tracers, plasma levels either plateaued or continued to rise. Efflux of all five tracers was significantly reduced in ascites tumor-bearing animals. Even at 6 h after i.p. injection, only 1% of the FITC-D tracers ≥20 kDa had accumulated in the plasma compartment, a 10- to 50-fold reduction as compared with control animals (Fig. 5). More than 95% of tracers ≥20 kD remained within the peritoneal cavities of the ascites tumor-bearing animals for at least 6 h (Fig. 5). However, a larger fraction of the 3 kDa FITC-D tracer escaped from the peritoneal cavities of ascites tumor-bearing animals, comparable to the levels found in normal animals. Tracer efflux was studied in animals that had received 10 μg of serotonin together with the i.p. injection of tracer. In these animals, efflux of the smaller two tracers was enhanced approximately 2-fold, and FITC-D concentrations in the plasma compartment were slightly increased above those found in normal controls. However, for FITC-D ≥ 70 kDa, efflux was identical to that observed in control mice. Finally, tracer efflux was studied in animals with artificial ascites (injected i.p. with 5 ml of 5% BSA along with FITC-D). Compared with normal animals, tracer efflux was decreased 2- to 12-fold, depending on the molecular weight of the FITC-D tracer. Three-Compartment Model. Using the experimental influx and efflux data, the three-compartment model depicted in Fig. 1, and the differential equations that describe that model (“Appendix”), we developed a general mathematical description of tracer flux among the several compartments; i.e., plasma, the peritoneal cavity, and the extravascular space of all other organs. Rate constants (Table 2) that satisfy these equations and that best fit our data were determined by computer, using an iterative, numerical-fitting approach (see “Appendix”). The influx and efflux curves calculated by using these kinetic parameters are plotted as solid lines in Figs. 3 and 5 and provide a good fit for most of the data. In accord with our immunofluorescence studies (Fig. 2) and quantitative tracer influx measurements (Fig. 3), the rate constants for the transfer of FITC-D from plasma to the peritoneal cavity (k3) increased substantially in ascites tumor-bearing and serotonin-injected animals, in comparison with normal control mice (Table 2; Fig. 6). Depending on tracer size, values for k3 increased 2- to 40-fold in MOT ascites tumors, 4- to 10-fold in TA3/St ascites tumors, and 2- to 10-fold following serotonin treatment. For comparison, in animals that had received 5 ml of 5% BSA to induce an artificial ascites, values of k3 increased 4-fold for the three larger FITC-D but for the two smaller FITC-D were identical to values in untreated control animals.
PERMSELECTIVITY OF PERITONEAL WALL

Fig. 3. Semilog plots describing the influx of FITC-D of varying sizes from the plasma to the peritoneal cavity at various intervals (abscissa) after i.v. tracer injection. FITC-D concentration (ordinate) is expressed as the fraction of injected dose (left axis) or percentage of injected dose (right axis) present either in the plasma (fxp) or in the peritoneal cavity (/x:"/). From left to right, columns represent normal control animals, MOT ascites tumor-bearing mice, TA3/St ascites tumor-bearing mice, normal mice that received 10 µg serotonin (5HT) i.p. at time t = 0 and normal mice that received i.p. 5 ml of 5% CB-SA at time t = 0. Each point represents 1 animal (3 to 5 animals for each time interval). Solid curves represent best computer-generated fit of the data, using the three-compartment model of Fig. 1 and the kinetic parameters presented in Table 2. Plasma (C), peritoneal fluid (M).

In agreement with our tracer efflux measurements (Fig. 5), values for k2, the rate constant for the transfer of tracer from peritoneal cavity to plasma, decreased from 5- to 50-fold in ascites tumor-bearing as compared with normal mice for all but the smallest FITC-D (Table 2; Fig. 7). In serotonin-treated animals, values of k2 actually increased ~2-fold for the smaller two FITC-D, but for the larger FITC-D were identical to values in untreated control animals. For comparison, in animals with artificial ascites, values of k2 decreased from 2.5- to 12.5-fold, depending on tracer size.

In general, values of both k1 and k2 fell progressively as the size of FITC-D increased (Table 2; Figs. 6 and 7). Values of the other rate constants (k3, k4 and k5) did not differ significantly among normal, ascites tumor-bearing, or serotonin-injected mice (Table 2), and their values were variably affected by tracer size (Fig. 8). Thus, k5, characterizing flux from plasma to other extracellular compartments, was constant for all tracers except that its value for the smallest tracer, 3 kDa FITC-D, was increased 20-fold (Fig. 8). This finding agrees with physiological measurements indicating that the 3 kDa FITC-D, unlike the others we tested, significantly permeates normal blood vessels having closed interendothelial junctions (9). Val-

Fig. 4. Sepharose CL-4B gel chromatography of aliquots of plasma and ascites fluid obtained from a MOT ascites tumor-bearing mouse 60 min following i.v. injection of 5000 kDa FITC-D. Fluorescence present in plasma (O) and ascites fluid (●) eluted from the Sepharose CL-4B column in fractions 23-25, identical to the elution pattern of 5000 kDa FITC-D prior to injection. Similar results were obtained with FITC-D of the other sizes used in this study (data not shown), confirming the stability of FITC-D in vivo reported by others (33).
PERMSELECTIVITY OF PERITONEAL WALL

Fig. 5. Semilog plots describing the efflux of FITC-D of varying sizes from the peritoneal cavity to the plasma at various intervals (abscissa) after i.p. tracer injection. As in Fig. 3, FITC-D concentration (ordinate) is expressed as the fraction of injected dose (left axis) or percentage of injected dose (right axis) present either in the peritoneal cavity or in the plasma. From left to right, columns describe normal control animals, MOT ascites tumor-bearing animals, TA3/St ascites tumor-bearing animals, normal mice that received 10 μg serotonin (5HT) i.p. at time t = 0, and normal mice that received 5 ml of 5% C-BSA i.p. at time t = 0. Each point represents 1 animal (3 to 5 animals for each time interval). Solid curves represent best computer-generated fit of the data, using the three-compartment model of Fig. 1 and the kinetic parameters presented in Table 2. Plasma (•), peritoneal fluid (○).

ues of the rate constant $k_5$, characterizing transport from other extravascular compartments into plasma, did not vary detectably with tracer size (Fig. 8). Values of the rate constant $k_5$, characterizing total tracer excretion, were unaffected by tracer size for FITC-D ≥ 70 kDa (Fig. 8). However, for the 3 and 20 kDa FITC-D, values of $k_5$ increased 33- and 17-fold, respectively, presumably reflecting the fact that dextrans in this size range are readily cleared from the kidney.

DISCUSSION

We measured the influx and efflux of a series of FITC-D into and out of the peritoneal cavity and, by computer, fit these data to a three-compartment model (consisting of the plasma, the peritoneal cavity, and the extravascular space of all other organs) that allowed us to describe mathematically the time-dependent distribution of dextran tracers in ascites tumor-bearing and control mice. Our data indicate that macromolecule (and presumably fluid) accumulation in tumor-bearing animals results both from increased influx and from retarded efflux.

The three-compartment model we used provided a satisfactory fit of our experimental data, using a minimum number of compartments (three) and parameters (five). In most instances, the computer simulations generated curves that conformed closely to the experimental data in the transient ($t < 15$ min), intermediate ($15$ min $< t < 120$ min), and steady-state ($t > 120$ min) portions of the $f_{xP}$ and $f_{xT}$ curves (Figs. 3 and 5). The error in the kinetic parameters $k_1$ to $k_5$ is estimated as ± 50%. This estimate is based on the observed changes in the numerically calculated time evolution of compartmental tracer concentrations as a function of changes in the trial values of the kinetic parameters.

In all four sets of mice (normal, ascites tumor-bearing, serotonin-injected, and normal with artificial ascites) $k_1$, the rate constant for passage of circulating tracers from the plasma into the peritoneal cavity, varied inversely with tracer size (Fig. 6); however, no absolute size barrier to macromolecular transport was found with tracers up to 5000 kDa and a Stokes' radius of ~40 nm. Depending on the size of the tracer, $k_1$ increased 2- to 40-fold above control values in mice bearing two different ascites tumors, 2- to 10-fold in mice that received an i.p. injection of serotonin to render their peritoneal wall blood vessels tran-
Table 2  Kinetic parameters $k_1$ to $k_5$ for FITC-D of varying size in control, ascites tumor-bearing, serotonin-injected animals and animals given injections i.p. of 5 ml of 5% BSA

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* Using the experimental influx and efflux data, the three-compartment model depicted in Fig. 1 and the differential equations that describe that model ("Appendix"), values for the rate constants that best fit the experimental data were determined by computer, using an iterative, numerical fitting analysis ("Appendix").

Fig. 6. A, dependence of the rate constant $k_1$ (influx from plasma into the peritoneal cavity) on mass of FITC-D tracer. B, values of the rate constant $k_1$ in ascites tumor-bearing or serotonin-injected animals relative to control animals ($k_1$ experimental/$k_1$ control) for FITC-D tracers of different molecular mass. Control (×), MOT (○), TA3/St (□), serotonin-treated (△), and C-BSA (□).

Fig. 7. A, dependence of the rate constant $k_5$ (efflux from the peritoneal cavity into the plasma) on mass of FITC-D tracer. B, values of the rate constant $k_5$ in ascites tumor-bearing or serotonin-injected animals relative to control animals ($k_5$ experimental/$k_5$ control) for FITC-D tracers of different molecular mass. Control (×), MOT (○), TA3/St (□), serotonin-treated (△), and C-BSA (□).
siently hyperpermeable, and at most 4-fold in mice given injections i.p. of BSA. For both tumor-bearing and serotonin-injected animals, values for \( k_i \) increased to an approximately equivalent extent for tracers of all sizes except the largest; influx of 5000 kDa FITC-D was also enhanced above normal control values, but to a significantly lesser extent than that of the other tracers.

The increased influx of macromolecules into tumor ascites is approximately equivalent in magnitude to that induced by a moderate dose of serotonin (10 \( \mu \)g; i.e., 0.5 mg/kg). However, tracer influx kinetics differed in serotonin-injected and ascites tumor-bearing mice. FITC-D accumulation peaked somewhat earlier in serotonin-injected animals. Moreover, enhanced influx continued for at least 6 h in ascites tumor-bearing animals, whereas that induced by serotonin was transient (limited to an interval of \( \sim 20 \) min) and is reportedly subject to tachyphylaxis (41–43). In the case of the smaller two FITC-D, \( k_i \) in artificial ascites-bearing mice was not increased above control values; however, for the larger three tracers, a moderate (4-fold) increase in \( k_i \) was observed. It is not clear why the presence of increased peritoneal fluid volume should lead to increased influx of the larger FITC-D. It is not attributable to endotoxin, contamination of BSA (44), in that endotoxin-free human albumin and even sterile saline or HBSS alone provoked a similar increase in \( k_i \) values.\(^5\) On the other hand, increased peritoneal fluid volume may be expected to retard macromolecular efflux (Table 2; Fig. 5), presumably by increasing the ratio of fluid volume to peritoneal surface area (22).

The passage of macromolecules from the plasma into most tissues, including the peritoneal cavity, is usually limited by the extent of vascular permeability. Therefore, it is likely that accumulation of increased FITC-D in ascites tumor-bearing animals reflects, at least in part, hyperpermeability of the blood vessels that line the peritoneal cavity. This conclusion was supported directly by fluorescence microscopy (Fig. 2). However, as noted earlier, \( k_i \) is a measure not only of vascular permeability but of a large number of other variables (45). In fact, it is very likely that interstitial tracer diffusion limited the influx of 5000 kDa FITC-D, as compared with smaller FITC-D, in animals with increased permeability of their peritoneal vessels; i.e., serotonin-injected and ascites tumor-bearing animals. Whereas 70 kDa FITC-D diffused rapidly and widely in the peritoneal walls and entered the peritoneal cavity within 1 min of i.v. injection, extravasated 5000 kDa FITC-D remained in the interstitium in the immediate vicinity of the vessels from which it had leaked for at least 15 min and was significantly slower to enter the peritoneal cavity (Figs. 2 and 3). In accord with this interpretation, others have found that interstitial diffusion of FITC-D within the mesentery behaves as an inverse function of tracer size (46). Glycosaminoglycans of the interstitial matrix, particularly hyaluronic acid, are thought to retard diffusion by spatially excluding tracer molecules (47, 48).

Efflux of all five FITC-D from the peritoneal cavity was significantly retarded in mice bearing both ascites tumors and the extent of retardation was significantly affected by tracer size. Comparing ratios of \( k_i \) in ascites tumor-bearing to those of normal mice (Fig. 7; Table 2), it is apparent that efflux was much more retarded in tumor-bearing animals for tracers \( \geq 70 \) kDa (10- to 50-fold) than for FITC-D of smaller size (0- to 5-fold). At least in part, retarded efflux in ascites tumor-bearing animals is attributable to the fact that i.p. injected tracers are immediately diluted in large volumes (2 to 10 ml) of protein-rich ascites fluid, as compared with normal mice whose endogenous peritoneal fluid volume is <0.2 ml. Thus, peritoneal efflux was also retarded (2.5- to 12.5-fold) in control animals given injections i.p. of 5 ml of 5% bovine serum albumin. This finding is in agreement with earlier data (16, 22) demonstrating significant retardation of tracer efflux in association with increased peritoneal fluid volume. However, the mere presence of increased proteinaceous peritoneal fluid did not fully account for the retarded efflux of ascites tumor animals (up to 50-fold), suggesting that other factors, such as lymphatic obstruction, may have contributed importantly in the latter groups (20, 21).

Efflux of FITC-D tracers from the peritoneal cavity to plasma was only slightly dependent on tracer size in control and serotonin-treated animals (Fig. 7). This finding is compatible with the fact that the stomata opening into the diaphragmatic lymphatics can accommodate molecules as large as 22 \( \mu \)m in diameter (49), much larger than that of any of the tracers studied here. In serotonin-injected animals, peritoneal efflux was modestly enhanced (~2-fold) for the 3 and 20 kDa tracers. The mechanism of this effect was not established but could reflect increased transport through the peritoneal mesothelium in addition to drainage via diaphragmatic lymphatics. In any event, this finding, together with the limited duration of the action of serotonin, accounts for the more rapid efflux of these tracers from serotonin-injected animals than from their ascites tumor-bearing counterparts (Fig. 5).

Our influx and efflux data in control animals are similar to those reported by others (32), who studied the distribution of 2-hydroxypropyl methyl acrylate copolymers (of similar size to our FITC-D) on unanesthetized animals. In contrast, Flessner et al. (29) have reported lower values for peritoneal clearance of FITC-D. A likely explanation for the discrepancy is that the animals in Flessner’s experiments were anesthetized; by depressing respiration and perhaps for additional reasons, anesthesia is known to retard peritoneal molecular transport (32).

The prominent inflection in the curves plotting \( k_i \) as a function of tracer size at a size of 70 kDa (Fig. 6A) is of interest and may indicate that more than a single mechanism of transport is involved; e.g., mechanisms that are size dependent (possibly diffusion or ultrafiltration) and others that are independent of size (possibly pinocytosis, vesicular transport, or convection). These data also support a maximal peritoneal pore radius of 5 nm (as determined from the intercept of the line before the break in slope) (31). The present study does not identify the anatomic site of these putative peritoneal pores. The break in slope is less abrupt in the ascites tumor-bearing animals, suggesting an altered porosity of the peritoneum in such animals. The analogous change in the slopes of the curves...
plotting $k_3$ as a function of tracer size may have a similar explanation (Fig. 7A). For example, the larger FITC-D may escape from the peritoneal cavity only via diaphragmatic lymphatics, whereas smaller FITC-D may also exit the peritoneum directly through the mesothelium covering other peritoneal surfaces.

To return to our original question, our data indicate that molecules as large as 5000 kDa are able to enter ascites tumor fluid without prior degradation (Fig. 4). Therefore, based on mass, fibrinogen (340 kDa) or other large plasma proteins should not be excluded from tumor ascites. However, measurements of molecular mass do not take into consideration molecular shape. Dextran form flexible chains in solution that are highly hydrated, loosely coiled, and nearly spherical in shape, whereas native globular plasma proteins are highly compact (50). Passage of FITC-D through the interstitial space of the peritoneal wall might be restricted because of their larger effective hydrodynamic radii relative to compact globular proteins of similar molecular mass (43). On the other hand, since dextrans are comprised of rather long chain polymers with only 6% degree of branching, the possibility exists that FITC-D may replete (i.e., "wiggle" or "squirm") through smaller pores than more globular proteins of equivalent mass. Therefore, comparisons between the transport of FITC-D and protein molecules should be made not on the basis of mass but rather on the basis of effective hydrodynamic radius. The effective hydrodynamic radii for the FITC-D used in this study are listed in Table 1. In contrast to dextrans or globular proteins, fibrinogen is a rod- or cigar-shaped molecule that behaves in solution like an ellipsoid of revolution with dimensions of 9 and 45 nm (51). Its hydrodynamic properties are consistent with an equivalent Stokes' radius of 8–10 nm, comparable to FITC-D of ~150 kDa, and therefore native fibrinogen would be expected to enter tumor ascites without difficulty.

At least one other physical property, charge, can be expected to influence molecular influx. The FITC-D studied here are neutral (52), whereas fibrinogen, with a pI of 5.5, carries a net negative charge at the near-neutral pH of plasma and tissues (53). Negative charge impedes extravasation of macromolecules in other vascular beds (54) and could retard the extravasation of fibrinogen from leaky peritoneal vessels in animals bearing ascites tumors. We are currently investigating this question.

Our data indicate that FITC-D tracers spanning a broad range of sizes enter into and accumulate in the peritoneal cavities of ascites tumor-bearing mice at significantly greater rates than in control animals. Based on fluorescence microscopy, this increased influx is attributable, at least in part, to increased vascular hyperpermeability. Increased influx, accompanied by retarded efflux from the peritoneal cavity, accounts for the accumulation of the protein-rich fluid that comprises tumor ascites. Vascular hyperpermeability and tracer influx and accumulation in ascites tumors appear to be similar to those reported in several of the same tumors grown in solid form, except that the latter are also characterized by extravascular fibrin deposits (1, 2, 6, 7, 9). One explanation for this difference is that the permeability properties of peritoneal wall vessels differ subtly from those of solid tumors with regard to the passage of fibrinogen. Another, perhaps more likely, explanation is that fibrinogen passes through peritoneal wall vessels with facility but is either not clotted or is rapidly degraded into small fragments before significant fibrin gel can accumulate. Experiments are in progress to distinguish among these possibilities.

Our findings may have important implications for tumor diagnosis and treatment (55). The impaired molecular efflux we have described may serve to retain therapeutic molecules locally and thus may importantly affect the kinetics of distribution when drugs or other molecules are administered by the peritoneal route to patients bearing ascites tumors (55). Moreover, the ability of monoclonal antibodies to enter solid or ascites tumors must depend heavily on the hyperpermeability of tumor vessels that we have reported here, because molecules of that size would not be expected to escape in significant quantity from normal vessels. We hope to design macromolecules whose properties favor their escape from tumor vessels and their accumulation in solid and ascites tumors. Such macromolecules could be useful therapeutically as carriers of drugs or toxins.

APPENDIX

A three-compartment model having the routes of tracer intercompartmental interchange indicated in Fig. 1, is described by the following differential equations (34):

$$\frac{df_x}{dt} = -(k_1 + k_2 + k_3) f_x + k_2 f_{xy} + k_3 f_{xx}$$  
(A)

$$\frac{df_{xy}}{dt} = k_1 f_x - k_2 f_{xy}$$  
(B)

$$\frac{df_{xx}}{dt} = k_3 f_x - k_4 f_{xx}$$  
(C)

where $f_x$ is the fraction of injected tracer in plasma, $f_{xy}$ is the fraction of injected tracer in the peritoneal fluid, and $f_{xx}$ is the fraction of injected tracer in all other extravascular compartments at any time, $t$. The kinetic parameters $k_1$ to $k_4$ are defined as in Fig. 1.

Influx Experiments. Initial trial values for the rate constants in Fig. 1 were determined by compartmental analysis (34). In this type of analysis, integration of the set of three simultaneous differential equations given above yields three equations for the fraction of tracer present in each of the three compartments (34). Each is mathematically expressed as the weighted sum of three exponentials since interchange of tracer is possible among all three compartments either by direct communication or indirectly via passage through the central (plasma) compartment. Equation D describes the fraction of tracer in the plasma compartment. Similarly, Equations E and F represent the fraction of tracer in the peritoneal fluid, $f_{xy}$, and in the extravascular space, $f_{xx}$, respectively.

$$f_x = H_1 e^{-r_1 t} + H_2 e^{-r_2 t} + H_3 e^{-r_3 t}$$  
(D)

$$f_{xy} = K_1 e^{-r_4 t} + K_2 e^{-r_5 t} + K_3 e^{-r_6 t}$$  
(E)

$$f_{xx} = L_1 e^{-r_7 t} + L_2 e^{-r_8 t} + L_3 e^{-r_9 t}$$  
(F)

We measured directly the fraction of tracer in two of these compartments, the plasma and the peritoneal cavity. Each set of experimental data for $f_x$ and for $f_{xy}$, under a particular set of experimental conditions, was then analyzed by curve peeling (34) to yield values for the respective coefficients and exponents given in Equations D and E. Curve peeling exploits the predominance of a single term of the three terms in the equation of the fraction of tracer during one of three consecutive experimental time periods: the steady state ($t > 120$ min), the intermediated (15 to 120 min), and the transient (0 to 15 min) periods.

The values of $g_1$, $g_2$, and $g_3$ generated from this analysis of the plasma and peritoneal data were averaged to yield the final values of the exponents $g_1$, $g_2$, and $g_3$ that were used, together with the coefficients $H_1$, $H_2$, $H_3$, $K_1$, $K_2$, $K_3$ to generate initial trial values for the rate constants, $k_1$, $k_2$, $k_3$, and $k_4$. The values of the rate constants were derived from complex expressions involving the coefficients $H_1$, $H_2$, $H_3$,
REFERENCES


PERMSELECTIVITY OF PERITONEAL WALL

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