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Targeting the *de novo* Biosynthesis of Thymidylate for the Development of PET Probe for Pancreatic Cancer Imaging

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Abstract

Development of cancer-specific probes for imaging by positron emission tomography (PET) is gaining impetus in cancer research and clinical oncology. One of the hallmarks of most cancer cell is incessant DNA replication, requiring continuous synthesis of nucleotides. Thymidylate synthase (TSase) is unique in this context, since it is the only enzyme in humans that is responsible for the *de novo* biosynthesis of the DNA building block 2'-deoxy-thymidylate (dTMP). TSase catalyzes the reductive methylation of 2'-deoxy-uridylylate (dUMP) to dTMP using (R)-N(5),N(10)-methylene-5,6,7,8-tetrahydrofolate (MTHF) as a cofactor. Not surprisingly, several human cancers over-express TSase, which makes it a common target for chemotherapy (e.g., 5-fluorouracil). We envisioned that [¹¹C]-MTHF might be specific PET probe to label cancerous cells. Using a stable radiotracer, [¹⁴C]-MTHF, we have initially found increased uptake by breast and colon cancer cell lines. In the current study, we examined the uptake of this radiotracer in human pancreatic cancer cell lines, MiaPaCa-2 and PANC-1, and found predominant radiolabeling of cancerous versus normal pancreatic cells. Furthermore, the uptake of the radiotracer is dependent on the intracellular level of the folate pool, cell cycle phase, expression of folate receptors on cell membrane, and co-treatment with the common chemotherapeutic drug methotrexate (MTX, blocking the biosynthesis of endogenous MTHF). These results point toward the potential for broad specificity of [¹¹C]-MTHF as PET probe, and the ability to control its signal using MTX co-administration.

Positron emission tomography (PET) is a non-invasive molecular imaging tool used to acquire images based on the biological or metabolic function of the tissues of interest. Thus, in medicine, these images can provide crucial spatial and pathological information to oncologists to arrange effectual treatment plans and to assess the progress of the current treatment plan.¹⁻³ As a result, PET has potential applications in oncology and cancer research, in which, positron emitting pharmaceutical probes are used in the imaging process. In general, positron-emitting radioisotope, such as ¹¹C, ¹⁸F, ¹³N, ¹⁴O, or ¹⁵O, is substituted for a stable isotope of the pharmaceutical compound, targeting the tracer accumulation in cancer cells, infection sites, or other tissues of interest.⁴⁻⁸

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Two common PET probes used in cancer imaging are [^{18}F]-fluorodeoxyglucose (FDG) and [^{18}F]-fluorothymidine (FLT). Based on the Warburg effect observed in cancer cells, the metabolic probe FDG is frequently used for cancer imaging. However, FDG is not a cancer specific probe. Its absorption is high in numerous normal tissues with high metabolic rates such as brain, heart, kidney or inflamed tissue.⁹ In contrast, FLT is a proliferation marker, which exploits the salvage pathway for the maintenance of intracellular thymidylate in certain cancers.¹⁰ Unfortunately, the detected levels of FLT are not always reliable, and the accuracy of both FDG and FLT is limited.^{11, 12} Another common drawback of fluorinated radiopharmaceuticals is their potential to defluorinate *in vivo*¹³ and their inhibitory activity in several biological pathways.

Compared to salvage pathway targeted by FLT, the *de novo* synthesis of thymidine (Fig.1) is ubiquitous in fast proliferating cells. FLT relay on the expression of thymine kinase (TdK), whereas the targeted *de novo* biosynthesis of thymidine relay on the enhanced expression of folate receptors (FRs) and the enzyme thymidylate synthase (TSase; EC 2.1.1.45). Consequently, developing a PET probe targeting the *de novo* synthesis will target different type of cancers and will complement FLT as a diagnostic tool. Recently, we reported the synthesis of [^{11}C] labeled (R)-N(5),N(10)-methylene-5,6,7,8-tetrahydrofolate ([^{11}C]-MTHF),¹⁴ the cofactor of TSase. Furthermore, in tissue culture studies, we reported the predominant labeling of cancer cells compared to their normal counterparts using [^{14}C]-MTHF, a stable isotopologue of [^{11}C]-MTHF.¹⁵

FRs and TSase play vital roles in the uptake and retention of the radiolabeled MTHF; FRs transport folates, including MTHF, into the cell where the enzyme TSase transfers the radiolabel methylene moiety to the precursor 2'-deoxyuridine-5'-monophosphate (dUMP). The catalytic conversion of dUMP to 2'-deoxythymidine-5'-monophosphate (dTMP), is a crucial step in the *de novo* biosynthesis of thymidine base in humans.¹⁶⁻¹⁸ Importantly, several cancers are known to overexpress FRs, including folate receptor-alpha (FR- α) and TSase.¹⁹⁻²¹ Specifically, the human pancreatic cancer cells overexpress FRs and TSase,²²⁻²⁴ suggesting these can serve as double selection targets for pancreatic cancer imaging. However, the cellular pool of folate is maintained in equilibrium with several other folate derivatives by folate recycling enzymes (e.g., dihydrofolate reductase, DHFR). Therefore the cells' dependency on the extracellular supply of MTHF depends on the DHFR activity and endogenous MTHF production. In this article we tested the uptake of MTHF by pancreatic cancerous vs. normal cell lines as a function of global intracellular folate pools and inhibited folate regenerating system.

EXPERIMENTAL PROCEDURES

Materials

^{14}C -formaldehyde, $^{14}\text{CH}_2\text{O}$ (50 mCi/mmol, 1-3% aqueous solution) was purchased from Moravsek Biochemicals (Brea, CA). (6S)-5,6,7,8-Tetrahydrofolate (THF) and N⁵,N¹⁰-methylene-5,6,7,8-tetrahydrofolate (MTHF) were a generous gift from Eprova-Merck. Preparation of [^{14}C]N⁵,N¹⁰-methylene-5,6,7,8-tetrahydrofolate ([^{14}C]-MTHF) was accomplished by mixing a solution of $^{14}\text{CH}_2\text{O}$ (50 μL) and a solution of THF (2 mg) in 100 μL solution of folate-stabilizing buffer. Folate-stabilizing buffer was prepared by dissolving

40 mg of ascorbic acid and 60 mg of trisodium citrate dehydrate in argon-purged 10 mL water.

Cell Culture

Pancreatic cancer (MIAPaCa-2 and PANC1) cell lines and primary pancreatic (H6c7) cells were kindly provided by Douglas Spitz and Joseph Cullen (University of Iowa), respectively. All cell cultures were conducted in 10-cm² culture plates in an incubator maintained at 37 °C under an atmosphere of 5% CO₂ and 95% air. The cells in log phase were passed or selected for uptake experiment when they were ~ 70% confluent as described previously.¹⁵ Primary pancreatic cells were grown in KSFM medium (Invitrogen) supplemented with EGF (2.5 µg), BPE (25mg), 50 IU/ml penicillin, and 50 µg/ml streptomycin. The cancer cells were initially grown in a regular, high-folate (2.3 µM) RPMI 1640 medium (HF, Invitrogen) supplemented with 10% heat inactivated FBS, 1% glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin. The cancer cells were then adapted in folate free RPMI 1640 medium (Invitrogen) (FF) supplemented 10% heat inactivated dialyzed FBS (Invitrogen), 1% glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin. Adaptation was started by placing the cells, originally grown in HF medium, in a medium containing 1:1 mixture of HF and FF media (FF1 population) for 3 days. FF1 population was then split 1:3 and passed in 100% FF medium for 3 days (FF2). Next passages in FF medium were maintained until a growth arrest stage was achieved (~FF6). For labeling studies, cell cycle analyses, and Western blots analyses, cells were when culture plates were around 65-75% confluent.

[¹⁴C]-MTHF uptake by cells

The uptake medium (30 ml) was prepared by supplementing FF medium with MTHF (20 µM final concentration) containing ~0.5 µCi of [¹⁴C] MTHF. For MiaPaCa-2 cancer cells, which showed loosely attached live cells in culture, the suspended cells were retrieved by collecting and centrifuging medium in 15 mL tubes. After removing the supernatant, the cell pelt was suspended in the labeling medium (3 mL per plate) and returned to the respective plate with attached cells. For Panc-1 cells, with no suspended cells, the growth medium was aspirated and the labeling medium (3 mL) was placed. For normal pancreatic H6c7 cells, the growth medium was aspirated, the cells were briefly (30 min) incubated with FF medium to remove any folate bound to the plasma membrane, and uptake medium was added. Cultures were incubated at 37 °C for 90 min with the uptake medium. Then, the culture plates were immediately placed on ice, the labeling media were aspirated and cells were washed twice with ice-cold 1 × PBS. The cells were detached with 0.25% trypsin-EDTA at room temperature, suspended in 0.5 ml of PBS and counted by using a Bio-Rad TC20TM automated cell counter. The cells were then transferred to scintillation vials, lysed in 0.5 ml of 0.5% SDS, mixed with 10 mL of scintillation fluid and counted in a liquid scintillation counter (LSC) for 5 min using the ¹⁴C window (0 – 156 kev). All the uptake experiments were conducted in two independent experiments with triplicates for each (6 samples total), and the flow cytometer measurements were consist of two independent experiments with duplicate for each (4 samples total). The error bars in all figures present the standard deviation of those measurements from the average.

Effect of methotrexate (MTX) treatment on the uptake of radiotracer

Cancer cells maintained in various passages including HF, and FF1-FF4, were tested for MTX-based enhancement of radioactivity uptake and retention. Growth media from the culture were aspirated and replaced with FF media supplemented with MTX at different concentrations and incubated for different time points. For time dependent experiments, the cells were treated with 10 nM MTX for 15, 30, 45, 60, 120 and 180 min. For dose dependent experiments, various concentrations of MTX (1, 3, 5, 10, 50, 100 nM) were used for 30-min incubation. After incubation, MTX containing media were removed and the cells were washed twice with 1x PBS. Then, the cells were immediately placed in the uptake media as described above. For control experiments, the cells were incubated with solvent (no MTX) followed by incubation in the labeling medium as described above.

Cell Cycle Analysis

Cancer cells maintained in various passages including HF, and FF1-FF4, were used for cell cycle analyses. The cells were harvested with trypsin, washed with cold 1x PBS twice and suspended at the density of 1×10^6 cells/ml in PBS. The cells were fixed overnight with ethanol at a concentration of 70% at -20°C . The cells were harvested from the alcohol suspension by centrifugation and re-suspended in the propidium iodide stain (1 mL). Propidium iodide (PI) staining buffer consisted of PI (5 $\mu\text{g/ml}$), RNase A (1 mg/ml), and glucose (1 mg/ml) in PBS. The stained cells were filtered through 70- μm nylon strainer and analyzed for DNA content on a FACScan flow cytometer (BD Biosciences) using FlowJo software. The data were analyzed using FlowJo 8.8.7 software.

Western Blot Analysis of folate receptor- α (FR- α) and TSase Enzyme

The culture medium of the cells in log phase was collected in 15 mL-tubes and centrifuged to collect the suspended cells. The detached cells were washed twice with cold PBS and harvested on ice with 200 μL of lysis buffer containing 1% Triton X-100, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS in 50 mM Tris, pH 8.0. To inhibit the proteasome degradation of proteins, a tablet of complete mini EDTA free protease inhibitor cocktail (Roche) was added in the lysis buffer. The extract was incubated on ice for 20 min, passed through a 25-gauge needle, and finally centrifuged at $15,000 \times g$ for 20 min at 4°C . The supernatant was removed in a 1.5 mL tube, and protein concentration was measured by using a BCA protein kit. A protein sample of 50 μg was loaded and electrophoresed on 10% SDS-polyacrylamide gels. Size standards from 10-250 kDa (BioRad Kaleidoscope) were included. Polypeptides were electro-transferred to nitrocellulose membranes. The membranes were blocked with OdysseyTM blocking buffer (Li-Cor) and probed with anti-human folate receptor or anti-human TSase antibody (Santa Cruz Biotechnology). For visualization, antispecies secondary antibodies labeled with AlexaFluor680 or AlexFluor800 were used. The membranes were blotted on a Li-Cor Odyssey scanner.

RESULTS AND DISCUSSION

Uptake of [^{14}C]-MTHF by primary and cancerous pancreatic cells

The radiotracer [^{14}C]-MTHF was used to distinguish between pancreatic cancer cells in comparison with normal pancreatic cells, and to test whether the radioactivity uptake can be

enhanced by treatment with MTX. We selected two pancreatic cancer cell lines (MIAPaCa-2 and PANC1) and compared the uptake of radiotracer with the normal pancreatic H6c7 cells. Both normal and cancerous cells were grown in their recommended media and selected for uptake experiment when they were in log phase (65-75% confluent). Prior to the experiment, the cells were incubated shortly with FF medium to wash off folates from media attached onto plasma membrane and then incubated in the labeling medium supplemented with 20 μ M MTHF containing 0.05 μ Ci of [14 C]-MTHF tracer for 90 min at 37 °C. The radiotracer taken up by the cells was determined by LSC and normalized to the total cell number and the initial dose of radioactivity using the given formula.

$$\text{Normalized uptake} = \frac{(\text{cpm recorded in cells})}{(\text{cpm added} \times \text{total cells} \times 10^{-6})}$$

As shown in Fig 2, primary pancreatic H6c7 cells accumulated less amount of the radiotracer relative to both cancerous cells. When compared with the normal pancreatic cells, the uptake of radiotracer by MiaPaCa-2 cells was about 3 fold higher. On the other hand, Panc1 showed about 7 fold more accumulation of tracers as compared to normal cells and almost double the levels observed in MiaPaca-2 cells line. These results indicate that [14 C]-MTHF can not only distinguish between normal versus cancerous cells, but may also be used to distinguish between these pancreatic cancer cell lines.

Uptake of [14 C]-MTHF by cancerous cells grown in FF medium

The regular HF medium for growing the MiaPaCa-2/Panc-1 cells contains ~2.3 μ M folates, which is approximately a 100 fold higher than the physiological concentrations found in humans.²⁵ Although, the culture media with such high non-physiological concentration of folate offer great growth advantages, yet they render the cells with abnormally high intracellular pools of folates. In such artificially high folate media, the expression of folate transporting proteins on the plasma membrane and folate transporting proteins including FR would be reduced. By lowering the intracellular folates close to the physiological levels (nM), we hope to be able to better assess the potential of the radiotracer *in vivo*. To test this hypothesis, we adapted both pancreatic cancer cell lines (i.e., MIAPaCa-2 and PANC1) in FF medium up to six passages (FF6). We observed continuous growth retardation starting from FF2 passage and complete arrest at FF6. Adaptation of normal pancreatic H6c7 was not successful as the cells' growth was arrested in FF medium at the FF1 stage.

Fig 3 shows the uptake of [14 C]-MTHF tracer in MIAPaCa-2 and PANC1 cells grown in HF and FF media. As expected, growing cells in FF medium enhanced the uptake of radiotracer. Enhancement was more prominent in MiaPaca cells where the uptake of radiotracer was almost 4 fold higher in FF4 passage than when the cells were grown in HF medium. In case of Panc-1 cell, enhancement in the uptake of radiotracer was only two-fold in the FF4 passage as compared to the cells grown in HF medium. When compared with the uptake by primary pancreatic cells, the MIAPaCa-2 cells in FF4 passage have almost twelve-fold enhancement and the PANC1 cells in FF4 demonstrated eighteen-fold enhancement (Fig 3).

This enhancement clearly indicated that the cells grown in low folate medium depends more on exogenous source of folate.

Effect of protein level expression of FR- α and TSase on the Uptake of [^{14}C]-MTHF

Western Blot was used to test the dependence of the uptake of [^{14}C]-MTHF on the expression of FR- α and TSase proteins in MIA PaCa-2 and Panc-1 cell lines growing under high folate and low folate conditions. The radioactive methylene in the labeled MTHF is trapped in cells by the reductive methylation of dUMP to form dTMP, which is catalyzed by TSase. The expression of this enzyme is also enhanced in FF medium (Fig. 4), which further enhance the uptake of [^{14}C]-MTHF radioactivity in the cells. As is shown in Fig 4, for MIA PaCa-2 cells, two bands were observed when the membranes were exposed to the anti-FR- α antibody (FL-257, Santa Cruz). The band at ~45 kD, corresponding to FR- α , remained expressed both in HF or FF media. Adaptation of MIA PaCa-2 cells to FF medium decreased the expression of this band sequentially, and about 78% of the protein, expressed in HF, is lost in the FF4 population. Interestingly, we observed another band at 52 kD whose expression increased in FF passages. We observed up to ~9-fold enhanced expression of this protein in FF4 when compared with the expression level in HF. That peak is likely to be a post-transcriptional modification of the mature FR receptor. Taken together, there is enhanced expression of FRs, when the cells were adapted to the FF medium (Fig. 4 C and D with 52+45 kDa bands combined) and this trend correlate positively with the uptake of radiolabeled external folate by MIA PaCa-2 cells.

Similar trends, with slight variation in the expression profile, were observed in case of Panc-1 cells, where the cells showed FR- α band at 45 kD until FF2 passage and then was completely absent in FF3 and FF4 passages. In contrast to MIA PaCa-2, in Panc-1 cells the band at 52 kD was constitutively expressed in all passages and became the only band after FF3 passages. The analysis showed an induced expression of TSase when both cell lines were adapted to the FF medium. In case of MIA PaCa-2 cells, up to 4-fold enhancement in the expression of TSase was observed in FF4 population, whereas, in Panc-1 cells, this enhancement was modest (i.e., ~ 2-fold) (Fig 4C&D). These data indicated that the enhancement of [^{14}C]-MTHF uptake in FF4 (Fig 3) depends on the expression of either folate receptors on the cell membranes or TSase expression in the cytoplasm.

Effect of intracellular recycling of folate on the Uptake of [^{14}C]-MTHF

The intracellular folate pool is maintained by the folate cycle (Fig 1), which involves catalytic conversion of folic acid and dihydrofolate (DHF) to tetrahydrofolate (THF) by dihydrofolate reductase (DHFR). The cytosolic serine hydroxymethyltransferase (cSHMT), then, catalyzes transfer of hydroxymethyl-group from serine to furnish the methylene (-CH₂-) moiety for the formation of MTHF from THF. Thus, the inhibition of DHFR is likely to reduce endogenous production of MTHF and increase the cell dependence on exogenous, radio-labeled MTHF. To test this hypothesis, we used MTX, a pM inhibitor of DHFR and common chemotherapeutic drug.²⁶ MIA PaCa-2 and PANC1 cells were treated with MTX (10 nM) for 30 min, before they were incubated with ^{14}C -MTHF uptake medium. Since uptakes of the radiotracer by the cells in FF1 and FF2 passages were almost similar to the uptake in HF medium, we compared the effects of MTX treatment in HF and FF media in

the passages FF3 and FF4. The cells in the same FF passages but without pretreatment with MTX were used as control.

The results are shown in Fig 5. Labeling of the cells grown in HF medium does not show significant enhancement due to MTX treatment. This phenomenon seems obvious when one considers the high concentration of folic acid in the HF medium (2.3 μ M), which competes for uptake of MTX by the same folate transporting proteins. The cells adapted to FF medium in the passage FF3 also did not show significant enhancement of the uptake of radiotracer. This observation might be attributed to sufficient concentration of intracellular THF and insufficient expression of folate transporting proteins, including FR- α in FF3 passages. The cells in FF4 passages, however, showed about 30% enhancements of radiotracer uptake in MIAPaCa-2 and Panc1 cells in response to the MTX treatment. The uptake enhancement of the radiotracer pointed toward the role of intracellular and extracellular pools of folates in the uptake of [14 C]-MTHF, which can be used as a marker to label the cells in high demand of folates. This outcome also supports the proposed mechanism of radioactivity trapping in the cell as presented above.

Cell Cycle Analysis

One of the hallmarks of cancer is aggressive proliferation, involving synthesis of new daughter DNA strands in the S phase of the cell cycle. Under such conditions, extensive synthesis of nucleotides including dTMP is required. Consequently, we undertook a study to find a correlation between MTHF uptake and the cell cycle. The cells were first fixed in alcohol as described in method section and then analyzed by using FACScan flow cytometer.

As apparent from Fig. 6, the population in G1 phase was increased with a concomitant decrease of the cells in S-phase. However, a small fraction of cells (~20%) was always present in G2/M phase, indicating a slow but steady growth rate. For breast and colon cells, we have reported that the cells passing through the S phase incorporate more MTHF.¹⁴ However in our current study with pancreatic cancer cells, the S phase of cells was gradually decreasing whereas G0/G1 phase was increasing from FF0 to FF4. However, the 14 C-MTHF uptake was higher in FF adopted cells in which FF4 had the highest uptake in both cell lines. Thus, the MTHF uptake does not directly correlate with the S phase of the cell cycle in MIAPaCa and PANC1 cell lines. This phenomenon probably result from a tradeoff between the cells adaption to low folate by moving to G0 phase on one hand, and increased dependence on the exogenous MTHF at the S phase on the other hand.

CONCLUSION

These findings demonstrate that [14 C]-MTHF radio-labeling of pancreatic cancerous cells is significantly larger than that for normal pancreatic cells. The uptake of the radiotracer increases with the dependence of the cells on exogenous MTHF associated with higher expression of folate receptors on cell membrane, expression of TSase, cell cycle phase, and MTX inhibition of endogenous production of MTHF. Apparently, the depletion of folate in media to their presumed *in vivo* level, led depletion of their intracellular level of folates, and the cells appear to depend more on extracellular folate resources, and thus to overexpress FR

and TSase to overcome folate starvation. In addition to endogenous folate depletion, the inhibition of intracellular folate recycling path by common chemotherapeutic agent MTX, an inhibitor of DHFR, also directed cells to rely more on extracellular folates and enhanced radiolabeling with [^{14}C]-MTHF. Overall, these findings point toward the specificity of the proposed PET probe, [^{11}C]-MTHF toward cancerous pancreatic tissues, and could potentially be exploited to image pancreatic cancer cells with low intracellular folate levels and over expressed FR and TSase. Additionally, since MTX is FDA approved, and in small dose could be administered to cancer patients prior to the PET procedure, it is possible that it could enhance the radiotracer uptake by pancreatic cancers and thus the S/N of the PET image.

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ABBREVIATION AND TEXTUAL FOOTNOTES

DHFR	dihydrofolate reductase
dTMP	2'-deoxythymidine-5'-monophosphate
dUMP	2'-deoxyuridine-5'-monophosphate
FDG	2'-deoxy-2'-[^{18}F]fluoroglucose
FF	folate free medium
FLT	3'-deoxy-3'-[^{18}F]fluorothymidine
FRs	folate receptors
MTHF	(<i>R</i>)- $\text{N}^5, \text{N}^{10}$ -methylene-5,6,7,8-tetrahydrofolate
MTX	methotrexate
PET	positron emission tomography
TdK	thymine kinase
TSase	thymidylate synthase.

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FIGURE 1.

Schematic representation of targeting the *de novo* synthesis of thymidine in folate overexpressed and DHFR inhibited cells by $[^{11}\text{C}]$ radiotracer. $[^{11}\text{C}]\text{-MTHF}$ is transported into cells via folate receptors (Fr- α) and $[^{11}\text{C}]\text{-radionuclide}$ is transferred into dUMP by TSase and converted into $[^{11}\text{C}]\text{-dTMP}$.

FIGURE 2.
[^{14}C]MTHF-uptake by normal vs. cancerous cells of pancreatic origin

FIGURE 3.

Uptake of [^{14}C]-MTHF by pancreatic cancer cells when grown in HF or FF medium compared to normal pancreatic cells

FIGURE 4.
Western Blot analysis of FR-α and TSase expression in (A) MIAPaCa-2 cancer cells (B) Panc-1 cancer cells (C) Analysis of MIAPaCa-2 (panel A) where FR-α (red) and TSase (blue) are normalized relative to β-actin. (D) Analysis of Panc-1 (panel B) where FR-α (red) and TSase (blue) are normalized relative to β-actin.

FIGURE 5.
Effect of MTX on the uptake of [^{14}C]-MTHF by pancreatic cancer cells

FIGURE 6.
Cell cycle analysis of (A) MIA PaCa-2 cells (B) Panc-1 cells grown in HF or FF medium.