QSYF Peptide Sequence Is Selected from Phage Display Libraries by Bovine IgG Contaminants in Monoclonal Antibody Preparations

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ABSTRACT

A consensus peptide sequence, QSYF, appears as an artifact during the mapping of monoclonal antibodies (MAbs) using a random peptide phage display library. Phage bearing this QSYF sequence were independently selected by four different laboratories screening separate MAb preparations with the same phage library. In each case, the QSYF sequence was selected in addition to a consensus sequence specific to the MAb. Phage that displayed the QSYF sequence were not bound by the MAb of interest, but rather bound to bovine IgG derived from the FBS present in the hybridoma growth media. The implications of this finding for the interpretation of phage library screening results and possible methods for the removal of bovine IgG from MAb preparations are discussed.

INTRODUCTION

Random peptide phage display libraries are used to identify peptide sequences that interact with specific proteins (5,17). This technique is commonly used to map epitopes bound by monoclonal antibodies (MAbs), and in cases where the epitope is comprised of discontinuous residues on the target protein, the amino acid sequences selected may also provide 3-D structure information about the antigen (2,6,8). The J404 phage display library expresses a random 9-mer peptide sequence as an amino-terminal extension to the pIII capsid protein of bacteriophage M13 (4,5). This library has been used in several studies to identify MAb-binding epitopes; in each case, the MAb selected phage for which a consensus sequence could be identified that matched the target antigen (2,4–8). In this study, the authors represent four separate laboratories, each of which has screened this random peptide phage display library against a different MAb. These MAbs were specific to unrelated antigens and represented a variety of Ig subtypes. However, in each case, the MAb selected phage bearing two separate consensus sequences: one sequence specific to the respective MAb, which could be mapped to an epitope on the antigen used to produce the MAb, and a second consensus sequence, QSYF. While searching for a

reason why we had all selected phage with the same sequence using seemingly unrelated MAbs, we discovered that all four MAb preparations had used hybridoma cells grown in media containing FBS, which contains bovine IgG (12). We hypothesized that the QSYF sequences were being selected from the library by bovine IgG, which was contaminating the MAb preparations. Purification of the MAb from the hybridoma culture supernatant by chromatography on immobilized protein G or protein A, which bind bovine, murine, and human IgGs (3,11), would lead to the presence of both the MAb and bovine IgG during the phage display selection.

MATERIALS AND METHODS

Materials

MAb F105 (16) is a human IgG₁ MAb directed against the envelope glycoprotein gp120 expressed on the surface of HIV and was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health, Bethesda, MD, USA (donated by Dr. M. Posner, Dana-Farber Cancer Institute, Boston, MA, USA). MAb F91 [donated by Dr. J. Robinson, Tulane University Medical Center, New Orleans, LA, USA (13)] is a human IgG₁

MAB directed against gp120 on HIV. MAb 449 [donated by Dr. D. Roos, Central Laboratory of the Netherlands Blood Transfusion Service, Amsterdam, The Netherlands (19)] was used in the initial phage display screening (7) but was unavailable in sufficient amounts for further ELISA-binding studies. MAb GZD1E8 (20), a mouse IgG₁, is specific for the major outer membrane protein of *Chlamydia pneumoniae*. MAb 4B4, a mouse IgG₃ against bovine rhodopsin (13), was purified from a hybridoma cell line (donated by Dr. R. Molday, University of British Columbia, Vancouver, BC, Canada). K42-41L (1) is a mouse IgG₁ MAB directed against bovine rhodopsin (donated by Dr. Paul Hargrave, University of Florida, Gainesville, FL, USA) that was grown by the National Cell Culture Center (Minneapolis, MN, USA) in serum-free media. The panel of mouse Ig isotypes was purchased from Zymed (San Francisco, CA, USA), and human polyclonal IgG was purchased from Sigma (St. Louis, MO, USA). Recombinant HIV gp120 (SF2) was a generous gift from Chiron (Emeryville, CA, USA). The J404 phage display library was produced in the filamentous phage M13KBst by inserting a degenerate (NNK)₉ sequence just downstream of the signal sequence coding region of the pIII capsid protein gene (4,5). The library has a complexity of approximately 5×10^8 unique phage. Peptide pep2Fg (SQSYPTRNS-amide) was purchased from Research Genetics (Huntsville, AL, USA) and further purified to >90% by reverse-phase HPLC. Alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L) was purchased from Bio-Rad Laboratories (Hercules, CA, USA), and rabbit anti-bovine IgG HRP-conjugate was purchased from Cappel (West Chester, PA, USA). FBS was obtained from HyClone (Logan, UT, USA).

ELISA

ELISAs were used to measure the binding of phage to Ab, peptide inhibition of phage binding to Ab, concentrations of bovine Ab, and capture of bovine Ab with phage. For phage-detection experiments, Ab was coated

directly onto microtiter wells (Immulon™ 2; Dynatech, McLean, VA, USA) by adding 0.1 mL 1–5 µg/mL solution and incubating overnight at 4°C. The concentrations of MAB include any contaminating bovine IgG. Wells were blocked with Blotto (5% nonfat dry milk, 0.5% TBST) for 3 h at room temperature. Phage was added at 5×10^8 to 1×10^{10} pfu/well, in a volume of 100 µL/well, and incubated overnight at 4°C. Plates were washed, incubated with rabbit anti-M13 serum (1:5000 dilution) for 2–4 h at room temperature, washed, incubated with goat anti-rabbit alkaline phosphatase-conjugated IgG for 1–2 h at room temperature, washed, and then developed by the addition of *p*-nitrophenyl phosphate (Sigma). For peptide-inhibition experiments, 100 µL of peptide at the indicated concentrations (see Figure 6) were added per well after the blocking step and incubated for 20 min at room temperature, followed by the addition of 50 µL of phage at varying concentrations overnight at 4°C. The remaining steps were as described above. For the detection of bovine IgG, the Ab sample of interest was coated directly onto microtiter wells (100 µL at 1 µg/mL total protein) and incubated overnight at 4°C. Wells were blocked with BSA for 3 h at room temperature. Rabbit anti-bovine IgG HRP-conjugate was added at a 1:1000 dilution in TBS either overnight or for 2–4 h at room temperature. The plates were washed and then developed using a 3,3',5,5'-tetramethylbenzidine (TMB)/hydrogen peroxide solution (Sigma). For the capture of bovine Ab with phage, phage was coated directly onto microtiter wells and incubated overnight at 4°C. The wells were blocked with BSA, washed, and 0.1 mL of the Ab samples was added to the wells at 2 µg/mL. After incubation overnight at 4°C, rabbit anti-bovine IgG HRP was added and detected as described earlier. Absorbance for HRP and alkaline phosphatase (A₄₅₀ and A₄₀₅, respectively) was determined using a microplate reader (Safire; Tecan Austria GmbH).

Phage Selection Experiments

For MAB F105, a “panning” protocol was used because only a small amount of MAB was available, and this

method uses less antibody than the bead attachment method. F105 was immobilized on 60-mm petri dishes (20 µg MAB/dish), incubated overnight at 4°C, washed, blocked with Blotto overnight at 4°C, and washed again. Phage library was added (1×10^{12} pfu), incubated overnight at 4°C, washed extensively (0.5% TBST), and eluted by the addition of rgp120 in 4× molar excess of MAB on the dish for 2 h at room temperature. Eluted phage was then amplified in *E. coli* strain K91, precipitated with 2.5% PEG/0.5 M NaCl, resuspended, and then used in subsequent rounds of selection (17).

MAbs 449, 4B4, and GZD1E8 were immobilized on Protein G Sepharose® beads (Amersham Biosciences, Piscataway, NJ, USA) using 300 µL total beads. The beads were divided into three aliquots for three rounds of selection. The phage library (1×10^{12} pfu) was added to the first aliquot and incubated overnight at 4°C. The bead/library mixture was placed in a column, washed extensively with ice-cold wash buffer (1% BSA, 0.5% TBST), and then eluted with 0.1 M glycine, pH 2.2. The eluted phage was neutralized immediately with 2 M Tris, pH 10, and then amplified as described earlier. Amplified phage was then combined with the second aliquot of beads, and the procedure was repeated until three rounds of selection were completed.

Analysis of Selected Phage

A dilution of the third-round elution was plated, and single plaques were excised. Phage minipreps were prepared by growing specific phage plaques overnight in Luria broth containing 75 µg/mL kanamycin. Phage was then isolated by PEG/NaCl precipitation, and their DNA was sequenced using a primer that anneals downstream of the unique 27-mer insert in the pIII gene, as previously described (4,5). For the F105 and bovine IgG selections, phage clones were prescreened by ELISA, and only clones that bound to the Ab sample were sequenced.

Protein G Isolation of Bovine IgG

A 2-mL slurry of Protein G Sepharose beads were placed in a col-

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Table 1. Sequences of Phage Display Peptides Selected by MAb F105

Phage Clone	MAb F105-Selected Phage Sequence
2Fg	S Q S Y P T R N S
26Fg	D Q S Y P A D A N
3Fg	G G S Q S Y P D L
7Fg	E D G G A Q S Y P
108Fg	W R T A P E S Y P
61Fg	V Q A T L Q S Y P
95Fg	I H Q S Y P D R G
91Fg	R E G A I Q S Y P
Consensus	Q S Y P
44Fg	R L T P E S D D R
53Fg	Q A R Y A K E P D
Specific Consensus	R X X P E P D
Phage display experiment was performed in the M. Teintze laboratory.	

umn, washed with 8 mL 100 mM glycine, 0.15 M NaCl, pH 2.5, and then washed with 100 mL PBS, pH 7.4. A total volume of 1 L 10% FBS in PBS was passed over the column, washed with 100 mL PBS, and eluted with eight 1-mL aliquots of 100 mM glycine, 0.15 M NaCl, pH 2.5. Eluate fractions were immediately neutralized with 1 M Tris buffer, pH 8.8, and their protein concentration was determined by measuring absorbance at 280 nm and using an extinction coefficient of 1.35 mL/mg-cm. Fractions containing the most protein (4 mg total) were pooled and then dialyzed against PBS at 4°C using Spectra/Por® membrane tubing, MWCO: 12–14 000 (Spectrum, Los Angeles, CA, USA). Verification of bovine IgG was obtained by visualizing the heavy and light chain bands on a reducing SDS-polyacrylamide gel (using Coomassie® blue staining) and showing the reactivity with anti-bovine IgG HRP by ELISA, as described earlier.

The isolated bovine IgG was also immobilized on Protein G Sepharose and used to screen the phage display library, as described earlier for the three MABs.

RESULTS

Four laboratories each independently screened the J404 nonapeptide phage

against the HIV envelope protein gp120 (16); 4B4, a mouse IgG₃ against bovine rhodopsin (13); 449, a mouse IgG₁ against human neutrophil flavocytochrome *b* (19); and GZD1E8, a mouse IgG₁ against the major outer membrane protein of *C. pneumoniae* (20). In each experiment, a consensus sequence of QSYP was found in addition to a different consensus that appeared specific for each MAB. Phage display selection against MAB F105 resulted in a number of phage clones that matched the QSYP consensus sequence (Table 1). QSYP-containing phage clones bound strongly to the MAB F105 preparation and the negative control MAB F91 preparation, as shown by phage clone 2Fg (Figure 1). MAB F91 was utilized as a negative control in these experiments because it was of the same species and isotype as MAB F105 and binds to a different epitope of the same antigen, gp120.

Table 2. Sequences of Phage Display Peptides Selected by MAB 4B4

MAb 4B4-Selected Phage Sequence	
	A G P P Y Q S Y P
	A M R D Y Q S Y P
	S Q S Y P D R
	S Q S Y P D
	Y M S Y P N R S A
	Y Q S Y P S R E H
Consensus	(Y/S) Q S Y P
Specific Consensus	E Q Q V S A T A Q
Phage display experiment was performed in the E. Dratz laboratory.	

display library using a different MAB: F105, a human IgG₁

The strong cross-reactivity of the MAB F91 preparation with phage clone 2Fg led us to use it as a prototypic “QSYP-binding” sample in additional binding studies. Many phage clones selected by MAB F105 also matched the consensus RXXPEPD (Table 1). Two examples in this group, 44Fg and 53Fg, were shown to bind specifically to MAB F105, but not to the negative control MAB F91 (Figure 1). The RXXPEPD sequence

Figure 1. Specificity of three phage clones isolated by selection with MAB F105. Plates were coated with MAB F105 and negative control MAB F91 at 0.1 µg/well. Phage clones were added at 1 × 10⁹ pfu/well. Parent phage M13KBst was a negative control. Phage binding was detected by rabbit anti-M13 antiserum, followed by AP-conjugated goat anti-rabbit secondary Ab. Absorbance values shown are the mean of duplicate samples with standard deviations shown as error bars.

Table 3. Sequences of Phage Display Peptides Selected by MAb 449

MAb 449-Selected Phage Sequence	
	YMSYP
	TDWHYQSYP
	TDLQYQRYP
	TDWQYQSYP
	YQSYP SREN
	YDHNYQSYP
	HMSYP
Consensus	YQSYP
Specific Consensus	GPQV
Phage display experiment was performed in the A. Jesaitis laboratory.	

mapped to a possible epitope on gp120 (J. Jacobs, unpublished data) that is consistent with the location of residues known to be escape mutations for F105 binding (18).

Phage screened against the MAb 4B4 sample also resulted in the selection of two consensus sequences (Table 2), with one consensus matching an epitope on the intradiskal face of

rhodopsin (8), while the other sequence (QSYP) could not be mapped to rhodopsin. Similarly, phage selected during screening with the MAb 449 (Table 3) produced a consensus sequence GPQV matching an epitope on cytochrome b, as well as sequences containing the QSYP motif (7). MAb GZD1E8 also selected phage with the QSYP consensus (Table

Table 4. Sequences of Phage Display Peptides Selected by MAb GZD1E8

Phage Clones	MAb GZD1E8-Selected Phage Sequence
38GZ	YQSFP
35GZ	YMSYP
48GZ	YQSYV
7GZ	SQSYPDR
43GZ	TSYQSRPT
37GZ	GKSQYESYP
42GZ	GAVSYESYP
27GZ	NKTAYESYP
39GZ	YMSYPNRSA
46GZ	AMRDYQSYP
26GZ	TDWQYQSYP
18GZ	QKHYYESYP
15GZ	GDVMYMSYP
Consensus	YQSYP
Phage display experiment was performed in the R. Morrison laboratory.	

4), in addition to phage with a consensus sequence that maps to the *C. pneumoniae* major outer membrane protein (R. Morrison, et al., unpublished data). The consensus sequence (QSYP) also appeared to include an additional Tyr residue (YQSYP) for MAbs 4B4, 449, and GZD1E8. Other sequences were isolated that did not correlate with either the QSYP consensus or the specif-

Figure 2. Detection of Bovine IgG in MAb preparations. Wells were coated with MAb samples (0.1 µg/well), and bovine IgG was detected by the addition of HRP-conjugated rabbit anti-bovine IgG. MAb K42-41L (grown in serum-free medium) and polyclonal mouse IgG1 (isolated from ascites fluid) were used as negative controls. Bovine IgG isolated from FBS was used as a positive control. Absorbance values shown are the mean of duplicate samples with standard deviations shown as error bars.

Figure 3. Binding of phage clones to MAb preparations. Wells were coated with MAb samples (0.1 µg/well); phage clones were added at 5 × 10⁸ pfu/well. Parent phage M13KBst was a negative control. Phage binding was detected by rabbit anti-M13 antiserum, followed by AP-conjugated goat anti-rabbit secondary Ab. Absorbance values are the mean of duplicate samples with standard deviations shown as error bars.

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ic consensus sequence, but these represented only a small percentage of the overall phage clones isolated.

The frequency with which the QSYF sequence was selected using both human and mouse MABs suggested that the QSYF-containing phage might bind a constant region shared by human and mouse Igs. ELISAs were used to screen a number of QSYF-containing phage clones against immunoglobulins of various isotypes, but these phage clones failed to bind either human IgG or mouse IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, IgA, and IgM (data not shown). This group of Igs included all the Ig classes and isotypes represented by the various MAB samples that were used to select the QSYF phage from the library. These negative results suggested that another component in the MAB preparation might be responsible for the selection of QSYF-containing phage.

It was determined that each MAB sample used for phage display selection had been produced using hybridomas cultured in media supplemented with 5%–10% FBS, which is known to contain bovine IgGs (12). The MABs had

been isolated using protein G affinity chromatography, a commonly used procedure that would enrich for all IgGs and not just the expressed MAB. We postulated that the QSYF sequence was not specific for the MAB of interest, but was instead selected by the contaminating bovine IgG. To test that possibility, we correlated the presence of bovine IgG in three of the MAB samples to the ability of that sample to bind phage containing the QSYF sequence. Rabbit anti-bovine IgG was used to test MAB preparations for the presence of bovine IgG. Figure 2 shows MAB samples 4B4, F91, and F105 cross-reacted well with the anti-bovine IgG, and the MAB sample GZD1E8 also showed a moderate cross-reaction. MAB K42-41L (14) was used as a negative control to show the background level of the experiment because it had been produced in serum-free media and should not contain bovine IgG.

The binding of QSYF-containing phage clones 35GZ and 26GZ to these MAB preparations was measured (Figure 3). Both QSYF-containing phage bound MAB preparations 4B4 and F91 at a high level, MAB preparation

GZD1E8 moderately, and MAB preparation K42-41L (bovine IgG-free) at background levels. These results show a direct correlation between the presence of bovine IgG in MAB samples (Figure 2) and the level of QSYF phage that binds to those samples (Figure 3). In addition, phage clones 35GZ and 26GZ, which had originally been selected using MAB GZD1E8, showed even greater binding to the MABs with the higher bovine IgG contamination, 4B4 and F91 (Figure 3). These data provided strong evidence that the QSYF sequence was being selected by contaminating bovine IgG rather than the MABs themselves.

To confirm that the QSYF-containing phage 26GZ bound bovine IgG, this clone was bound to wells of an ELISA plate at various concentrations and used to capture bovine IgG from MAB samples (Figure 4). The captured bovine IgG was detected with rabbit anti-bovine IgG HRP conjugate. The QSYF-containing phage 26GZ bound bovine IgG from MABs 4B4 and F91, two samples contaminated with bovine IgG, but control M13 phage lacking the QSYF sequence did not bind bovine

Figure 4. Capture and detection of Bovine IgG contaminating MAB preparations by QSYF phage clones. Phage clones were placed in wells at increasing concentrations. MAB samples were added (0.2 µg/well), and the bovine IgG in the MAB sample was captured by the QSYF-containing phage and detected by rabbit anti-bovine IgG-HRP. MAB preparations were tested against both negative control parent phage (M13KBst) and 26GZ (QSYF-containing phage clone). Absorbance values are the mean of duplicate samples with standard deviations shown as error bars.

Figure 5. Binding of QSYF phage clones to increasing concentrations of serum-free MAB K42-41L and bovine IgG. MAB K42-41L and bovine IgG were placed in wells (0.1–0.5 µg/well). Phage clones were added at 5×10^8 pfu/well, and phage binding was detected by rabbit anti-M13 antiserum, followed by AP-conjugated goat anti-rabbit secondary Ab. Parent phage M13KBst was used as a negative control. The legend shows symbols corresponding to the different combinations of MAB K42-41L (serum free) and bovine IgG with QSYF-containing phage clones (7GZ and 35GZ) and negative control M13KBst. Absorbance values are the mean of duplicate samples with standard deviations shown as error bars.

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Figure 6. Peptide inhibition of QSYP-containing phage clones binding to bovine IgG. Bovine IgG was coated on the plate (0.3 μg/well). Peptide was added to wells at various concentrations for 20 min at room temperature, followed by the addition of phage clones at 5×10^8 pfu/well incubated overnight at 4°C. Phage binding was detected by rabbit anti-M13 antiserum, followed by AP-conjugated goat anti-rabbit secondary Ab. Symbols correspond to different combinations of negative control peptide pep44Fg (RLTPEPDD) and QSYP-containing peptide pep2Fg (SQSYPTRNS) inhibiting the binding of two QSYP-containing phage clones, 13D (SQSYPTDR) and 2Fg (SQSYPTRNS). Absorbance values are the mean of triplicate samples with standard deviations shown as error bars.

IgG. Bovine IgG was not captured from the MAb produced in serum-free media, K42-41L, as expected.

To demonstrate that bovine IgG contamination could have originated from serum in the culture media, bovine IgG was isolated from the FBS by protein G affinity chromatography. When the purified bovine IgG was analyzed on a reducing SDS-polyacrylamide gel, only two bands were seen, which corresponded to the expected migration distances of the heavy and light chains of immunoglobulin (data not shown). The purified bovine IgG reacted with rabbit anti-bovine IgG using ELISA (Figure 2) and bound specifically to QSYP-containing phage (Figure 5). A synthetic peptide corresponding to the phage clone 2Fg, SQSYPTRNS, was synthesized and tested to determine its ability to inhibit binding of QSYP-containing phage to MAb samples. Peptide pep2Fg inhibited the binding of QSYP-containing phage clones 2Fg and 13D (compared to no inhibition observed with control

peptide pep44Fg-RLTPEPDD) with a half-maximal peptide effect in the nanomolar range (Figure 6). These data show that the QSYP sequence itself was responsible for the binding of the phage to the bovine IgG in the MAb samples and that this binding was not dependent on attachment to the phage.

To further test our hypothesis, the bovine IgG isolated from FBS was bound to Protein G-Sepharose beads and used to screen the phage display library in the same manner as had been used for MAb samples 4B4, 449, and GZD1E8. After three rounds of selection, 60 clones were screened with ELISA for binding to purified bovine IgG, and 12 clones were sequenced. Seven of these were found to contain sequences matching the (Y/S)QSYP consensus (Table 5); the remaining sequences did not fit any particular pattern (data not shown).

DISCUSSION

Four MAb preparations with specificity for different antigens selected phage with the same QSYP consensus sequence from a random peptide phage display library. Phage clones containing this sequence bound to the MAb

Table 5. Sequences of Phage Display Peptides Selected by Bovine IgG

Phage Clones	Bovine IgG-Selected Phage Sequence
3,8,30, and 31BG	Y Q S Y P S R E H
21BG	S Q S Y L D R
37BG	Y M S Y P
41BG	S Q S Y P D T
Consensus	(Y/S)Q S Y P
Phage display experiment was performed in the M. Teintze laboratory.	

preparations in proportion to the amount of bovine IgG that contaminated the samples. Many MAbs are produced using hybridoma cell lines grown in media containing up to 10% FBS, which contains significant amounts of bovine IgG (12). A widely used protocol for purifying the MAbs from the culture supernatant involves affinity chromatography on Protein G-Sepharose beads, which will co-purify any bovine IgG in the medium (3,11).

The data presented here demonstrate that bovine IgG can select QSYP-containing phage from a peptide display library. Whether a sufficient number of such phage clones are selected to result in a secondary consensus sequence appearing in the alignments presumably depends on the relative amount of the bovine IgG contamination in the purified MAb preparation. A lower yield of MAb in a hybridoma cell supernatant could result in a proportionately higher fraction of bovine IgG contamination after protein G affinity chromatography purification (15). We present these results to caution other investigators who may find such bovine IgG-selected phage sequences and to suggest that, in some instances, it might be worthwhile to use either a further purification step to remove bovine IgG from the MAb preparation or to grow the hybridomas in serum-free or bovine IgG-depleted media (9). There are a number of methods by which bovine IgG can be removed from the MAb sample. A more specific affinity purification method could be used on the culture supernatant, such as immobilizing the target molecule of the MAb or using protein L, which is known not to bind bovine

IgG (10). The phage library could be preincubated with immobilized bovine IgG to remove phage clones containing peptides specific for bovine IgG. The hybridoma supernatant could also be depleted of bovine IgG using an immobilized rabbit anti-bovine IgG column.

It is not known where the QSYP sequence binds to the bovine IgG molecule. Presumably, the bovine IgG contamination is polyclonal in nature, and the QSYP sequence binds to a constant region on the antibody, but the interaction has not been characterized further as to isotype specificity or location of the binding site. Phage display libraries may contain different populations of peptides or display them differently, depending on the length of the peptide, whether it is linear or constrained in a loop, and on what phage protein it is displayed. Therefore, bovine IgG might select different sequences from other phage libraries than the one reported here.

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