Amyloid Form of Ovalbumin Evokes Native Antigen-specific Immune Response in the Host

PROSPECTIVE IMMUNO-PROPHYLACTIC POTENTIAL

Received for publication, July 6, 2014, and in revised form, December 3, 2014 Published, JBC Papers in Press, December 15, 2014, DOI 10.1074/jbc.M113.540989

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Background: Amyloids have recently been found to be reversible and dynamic. They release the precursor peptide/protein in a slow and sustained manner.

Results: Antibodies produced in response to amyloid immunization could recognize native antigen.

Conclusion: OVA amyloids release proteins harboring native antigen epitopes.

Significance: The slow and sustained release of native antigen from amyloids makes them potential candidates for inducing a protective antibody response.

Amyloids are highly organized protein aggregates that arise from inappropriately folded versions of proteins or polypeptides under both physiological as well as simulated ambiances. Once thought to be irreversible assemblies, amyloids have begun to expose their more dynamic and reversible attributes depending upon the intrinsic properties of the precursor protein/peptide and experimental conditions such as temperature, pressure, structural modifications in proteins, or presence of chemicals in the reaction mixture. It has been repeatedly proposed that amyloids undergo transformation to the bioactive peptide/protein forms under specific conditions. In the present study, amyloids assembled from the model protein ovalbumin (OVA) were found to release the precursor protein in a slow and steady manner over an extended time period. Interestingly, the released OVA from amyloid depot was found to exhibit biophysical characteristics of native protein and reacted with native-OVA specific monoclonal as well as polyclonal antibodies. Moreover, antibodies generated upon immunization of OVA amyloidal aggregates or fibrils were found to recognize the native form of OVA. The study suggests that amyloids may act as depots for the native form of the protein and therefore can be exploited as vaccine candidates, where slow antigen release over extended time periods is a pre-requisite for the development of desired immune response.

There is a general consensus that under destabilizing conditions, abnormally folded intermediates of several proteins have a strong tendency to self-aggregate into a polymeric amyloid fibril (1, 2). Various proteins and peptides have been found to form amyloids in diverse conditions implying that amyloid formation is a generic feature of peptide and proteins (3–5). The amyloid fibrils harbor a core formed by cross-β-structures where β-strands are oriented perpendicularly to the main fibril axis (1, 6). The β-cores can bind to the amyloid binding dyes thioflavin T (ThT)4 and Congo Red (7, 8). Amyloidal bodies have been associated with the pathogenesis of several neurodegenerative diseases such as Alzheimer, Parkinson, or Creutzfeldt-Jakob disease etc. (9–11). In downward contrast, a few naturally existing amyloids have been found to perform non-pathogenic rather beneficial functions that are crucial for the survival of the host, such as curli fibrils expressed by Escherichia coli assist in cell-cell contact (12), amyloidal protein of chorion protects oocyte, and developing embryo of silkworm (13). Likewise, fungal prions including yeast and HETs prions in certain cases enhance survival of the host (14–16), whereas Pmel17 amyloid promotes skin pigmentation in humans (17). Also, peptides and protein hormones present in the pituitary secretory granules have been found to carry amyloid-like cross-β-sheet rich conformation (18). Amyloids have also been associated with transfer of genetic information or synaptic changes linked to memory (19–22). Moreover, some recent findings enumerate that artificially or in vitro synthesized amyloids can also perform beneficial biological activities in vivo (23, 24).

Amyloid fibrils classically viewed to be highly stable structures capable of withstanding perturbing environmental conditions have begun to be realized as more of dynamic entities that may revert back to their native form. Variations in temperature (25) and pressure (26, 27), contamination by chemical denaturants (28), and structural modifications in proteins (29, 30) have been found to destabilize amyloid fibrils. Employing hydrogen/deuterium exchange experiments, Carulla et al. (31) have inge-

1 Supported by the Council of Scientific and Industrial Research (CSIR), India, Senior Research Fellowship (SRF).

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4 The abbreviations used are: ThT, thioflavin T; DTH, delayed type hypersensitivity; PB, phosphate buffer; CD, circular dichroism; ELISA, enzyme linked immunosorbent assay; Th1, type 1 T helper; Th2, type 2 T helper.
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Naturally demonstrated that monomeric Src homology domain 3 molecules incorporated into Src homology domain 3 amyloid fibrils continuously recycle between the fibril and the monomer state by a dissociation/re-association mechanism at the fibril ends. The recent findings by Kardos et al. (25) enumerated dissociation of β2-microglobulin fibrils to be a reversible and dynamic process reaching equilibrium between fibrils and monomers following time kinetics of the order of a few minutes. In an earlier study, β2-microglobulin fibrils were found to completely dissociate to monomeric form in dimethyl sulfoxide (28). Binger et al. (30) have shown that hydrogen peroxide-mediated oxidation of methionine residues in the preformed apoCII fibrils reverses their assembly and dissociates the monomer in a time-dependent manner. Also, approximately 2–4% of the monomers have been found to remain unpolymerized at the culmination of a process (29). Flynn et al. (26) have shown that hydrogen peroxide-mediated oxidation of methionine residues in the preformed apoCII fibrils reverses their assembly and dissociates the monomer in a time-dependent manner. Also, approximately 2–4% of the monomers have been found to remain unpolymerized at the culmination of a process (29).

In the present study, OVA (a non-inhibitory member of the serpin protein family) was taken as a model protein for the synthesis of amyloid bodies. OVA was continuously agitated at varying pH conditions (pH 2.5, 7.0, and 10) and the aggregates obtained after various incubation periods were characterized by turbidity measurements, Rayleigh scattering studies, ThT, and Congo Red binding, CD spectral measurements etc. Finally, the formation of fibrils was confirmed by transmission electron microscopy. Furthermore, release of OVA from various aggregates was monitored over an extended time period followed by assessing the state of released product and its conformational integrity. The amyloidic aggregates formed at either pH 2.5 or 7.0 were then studied for their immunological properties. The antibodies generated in response to the immunization and those incorporated into fibrils. Moreover, in a few recent reports non-fibrillar as well as fibrillar aggregates generated from the same protein have been found to exhibit varied release kinetics depending upon the compactness and ordered nature of the aggregates (23, 24). Aggregates obtained at earlier incubation time periods have been found to attain rapid saturation in the release profile in comparison to fibril species formed at later stages. Release of monomers from some of the aggregates could reach saturation only after a period of over 15 or 30 days, which indicates that in such cases equilibrium between the fibril or other amyloidic aggregates and the released monomers are attained after long time periods and therefore the aggregates could subsequently release the monomeric protein in a sustained manner over an extended time course. Although overwhelming information is available regarding conformation of amyloid fibrils and release of proteins from them, there remains a paucity of reports on the conformation of the released proteins and peptides.

In the present study, OVA (A2512), thioflavin T, Congo Red, and fetal calf serum were purchased from Sigma. IgG2a (R35–95) isotype control was procured from eBiosciences (San Diego, CA). IgG1 and IgG2a isotypes (550487), and cytokines viz. IL-4, interferon-γ, and IL-12 cytokine estimation kits were procured from BD Biosciences, OptEIA (Franklin Lakes, NJ). RPMI 1640, antimycotic solution, and plasticwares were purchased from BD Biosciences (San Diego, CA). Anti-OVA monoclonal antibodies 2D11 and 3G2E1D9 were purchased from Santa Cruz Biotechnology, Inc.

**Fibril Formation**—OVA was dissolved in PBS (pH 7.0) (24) or glycine–HCl (pH 2.5) or glycine–NaOH (pH 10) buffers (carrying 0.01% azide) at a concentration of 1 mg/ml and incubated at room temperature under continuous agitation (at 90 rpm). Aliquots collected at various time points were pelleted down at 15,000 × g for 15 min using Sigma 3K30 (Germany) microcentrifuge. Under these conditions, monomeric OVA does not sediment and thus remained in solution only. Fibril formation was established by ThT and Congo Red binding assays and transmission electron microscopy. There was no aggregate formation upon incubation of OVA at pH 10 (data not shown).

**Rayleigh Scattering Measurements**—Rayleigh scattering measurements were performed on a Hitachi F-4500 fluorescence spectrophotometer at room temperature using a cell with a 1-cm light path. The excitation and emission wavelengths were both set at 350 nm, and the slit length was 5 nm.

**Turbidity Measurements**—For turbidity measurements, the incubated samples collected at various time points were monitored by UV absorbance at 350 nm using a PerkinElmer UV-visible spectrometer model A25 in a 1-cm path length cuvette at room temperature.

**Congo Red Binding Studies**—Congo Red solution (20 μM) prepared in phosphate buffer (PB) (pH 7.4) (using a stock solution (1 mM) of Congo Red prepared in ethanol) was incubated with 100 μg of aggregates for 30 min at room temperature. UV absorbance was measured in the spectral range at 300–700 nm using a PerkinElmer UV-visible spectrometer model λ25.

**ThT Binding Studies**—OVA aggregates (100 μg) obtained at various time points were incubated with 30 μM ThT solution (30 μl of 1 mM ThT stock solution) at room temperature and fluorescence was measured on Hitachi F-4500 fluorescence spectrophotometer after 30 min. The bound ThT amyloid was excited at 450 nm and spectra were recorded from 460 to 560 nm. The excitation and emission slit widths were fixed at 5 and 10 nm, respectively.

Interaction of various amyloid species obtained at various time points with ThT was also assessed by fluorescence microscopy. Incubated OVA aggregates obtained at various time points, viz. days 2, 4, 7, 10, and 15 were pelleted down at 15,000 × g for 15 min. The pellets obtained were incubated with 20 μM ThT for 30 min at room temperature and then trans-

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—All the reagents used were of the highest purity available. OVA (A2512), thioflavin T, Congo Red, and fetal calf serum were purchased from Sigma. IgG2a (R35–95) isotype control was procured from eBiosciences (San Diego, CA). IgG1 and IgG2a isotypes (550487), and cytokines viz. IL-4, interferon-γ, and IL-12 cytokine estimation kits were procured from BD Biosciences, OptEIA (Franklin Lakes, NJ). RPMI 1640, antimycotic solution, and plasticwares were purchased from BD Biosciences (San Diego, CA). Anti-OVA monoclonal antibodies 2D11 and 3G2E1D9 were purchased from Santa Cruz Biotechnology, Inc.
ferred onto a glass slide to be analyzed under fluorescence microscope (Axio, HBU 50/AC; Zeiss, Gottingen, Germany).

CD Measurements—A JASCO spectropolarimeter (J-815) was used for circular dichroic measurements using quartz cell with 0.1-cm path length. The temperature was controlled at room temperature using a Peltier Thermostat with Multitech water circulator and the instrument was calibrated using D10-camphor sulfonic acid. A scan speed of 100 nm/min and response time of 2 s were used for spectra collection. Scans were performed for each sample in the range of 200–250 nm with final protein concentrations being 200 μg/ml.

Transmission Electron Microscopy—For electron microscopy, 6 μl of protein sample (100 μg) was spread on a carbon-coated copper grid that was further negatively stained with 2% (w/v) uranyl acetate. The grid was examined under a JEOL transmission electron microscope operating at an accelerating voltage of 200 kV.

In Vitro Release Kinetics—OVA (1 mg/ml of PBS, pH 7.0, and glycine-HCl, pH 2.5) samples incubated for fibrillation were withdrawn at various time points. The amyloids formed were isolated by pelleting at 15,000 × g for 15 min. The pellets obtained were washed three times with PB (pH 7.4) and re-suspended in PB (pH 7.4). The kinetics of OVA release into PB (pH 7.4) was monitored spectrophotometrically at 280 nm (24) as well as by determining intrinsic fluorescence measurements (32). The supernatant obtained after centrifugation was analyzed at various time points for approximately 15 days using PerkinElmer UV-visible spectrometer model λ25 and Hitachi F-4500 fluorescence spectrophotometer, respectively.

Size Exclusion Chromatography—Five milliliters of the sample containing OVA released from various aggregates after 12 days (approximately 10 mg) was applied to a 83-cm long column with an internal diameter of 2.3 cm, filled with about 340 ml of preswollen Bio-Gel P10 (Bio-Rad), and equilibrated with 20 mM phosphate-HCl buffer (pH 3.0) containing 18 mM NaCl. A scan speed of 100 nm/min and a flow rate of 35 ml/h and 1.0-ml fractions were collected. Absorbance of the collected fractions was measured by a PerkinElmer UV-visible spectrometer model λ25 and Hitachi F-4500 fluorescence spectrophotometer, respectively.

Western Blot Analysis of the OVA Released from Various Aggregates—Released OVA from various aggregates after various incubation periods was resolved by electrophoresis on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto PVDF membrane. After blocking in 5% nonfat dry milk prepared in phosphate-buffered saline (PBS) with Tween (PBST), the membrane was washed three times with PBST and incubated for 2 h at 37 °C with either anti-OVA polyclonal or 3G2E1D9 monoclonal antibody. After incubation and stipulated washing steps, the membrane was incubated with horse-radish peroxidase-conjugated goat anti-mouse antibody (1:5000) for 1 h at 37 °C. Finally, post washing, the bands onto the membrane were developed by enhanced chemiluminiscence (ECL) using ECL kit (Bio-Rad).

Animals—Inbred female BALB/c mice (6–8 weeks old, 20 ± 2 g) were obtained from the Animal House Facility of the Institute. The BALB/c mice were housed in commercially available polypropylene cages and maintained under controlled temperature conditions on a 12-h light/dark cycle and had free access to food and water ad libitum. OT-I and OT-II mice originally obtained from The Jackson Laboratory were a gift from Dr. Satyajit Rath (National Institute of Immunology, New Delhi, India). OT-I and OT-II mice were bred and maintained in the Animal House Facility of the National Institute of Immunology (New Delhi, India). All the animal experiments were performed according to the National Regulatory Guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Mode and Schedule of Immunization—Animals were immunized subcutaneously in the lower abdominal region aseptically with 50 μg of OVA amyloid bodies (obtained from fibrillation reaction at various time points). A booster was given 3 weeks after the first immunization with 25 μg of the corresponding form of protein fibril.

Collection of Sera—The blood was collected from mice after 5 days of last booster. Sera were separated from the clotted blood by centrifugation at 1500 × g for 10 min at 4 °C. Finally, the supernatant was collected for ELISA analysis and purification of polyclonal antibodies.

Determination of Antigen-specific Total IgGs by ELISA—Antigen-specific total IgGs against the native and as well as OVA aggregates were determined in the sera of mice immunized with various aggregates of OVA formed at various time points following the protocol as described elsewhere (33). Briefly, 100 μl (2 μg) of native OVA as well as OVA aggregates obtained at various time points were dissolved in carbonate/bicarbonate buffer (0.05 M, pH 9.6) and poured into 96-well microtiter plates that were further incubated overnight at 4 °C. The plates were then incubated with serially diluted sera at 37 °C for 2 h, after the usual washing and blocking steps. Furthermore, the plates were washed again and 100 μl of (1:5000 dilution of stock) horseradish peroxidase-conjugated goat anti-mouse antibodies were added to each well and the plates were incubated at 37 °C for 1 h. After the usual plate washing, 100 μl of substrate solution (6 mg o-phenylenediamine) in 12 ml of substrate buffer with 5 μl of 30% H2O2 was added to the wells and the plates were finally incubated at 37 °C for 40 min. The reaction was terminated by the addition of 50 μl of 7% H2SO4. The absorbance was read at 490 nm with a microtiter plate reader (Bio-Rad).

ELISA of OVA Released from Various Aggregates—Briefly, 96-well microtiter plates were incubated overnight with 100 μl (2 μg) of OVA released from various aggregates in carbonate/bicarbonate buffer (0.05 M, pH 9.6) at 4 °C. After the usual washing and blocking steps, the plates were finally incubated with serially diluted anti-native OVA-specific polyclonal and 2D11 monoclonal antibodies at 37 °C for 2 h. After washing the plates, 100 μl of (1:5000 dilution of stock) horseradish peroxidase-conjugated goat anti-mouse antibodies were added to each well and the plates were incubated at 37 °C for 1 h. Substrate solution (100 μl) was added to the wells after the usual plate washing, which were finally incubated at 37 °C for 40 min. For terminating the reaction, 50 μl of 7% H2SO4 was added to the wells. The absorbance was read at 490 nm with a microtiter plate reader (Bio-Rad).
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Determination of Antibody Isotype in Sera of Immunized Mice—Sera from mice immunized with day 4 and 7 aggregates formed at both pH 2.5 and 7.0 were analyzed for antibody isotypes using the protocol described elsewhere (34). Briefly, 2 μg (100 μl) of antigen in carbonate/bicarbonate buffer (0.05 M, pH 9.6) was added to 96-well microtiter plates that were incubated overnight at 4 °C. After washing and blocking steps, the plates were incubated with serially diluted sera at 37 °C for 2 h. After washing of the plates, 100 μl of (1:5000 dilution of stock) goat anti-mouse anti-IgG1, and IgG2a antibodies were added in each well and incubated for 1 h at 37 °C. Plates were then washed and 100 μl of (1:5000 dilution of stock) rabbit anti-goat antibodies were added to each well and each plate was incubated at 37 °C for 1 h. After further washing of the plates, 100 μl of substrate solution was added to the wells and the plates were finally incubated at 37 °C for 40 min. The reaction was stopped by the addition of 50 μl of 1 N H2SO4. The absorbance was read at 490 nm with a microtiter ELISA plate reader (Bio-Rad).

Measurement of DTH Response—The animals were immunized with day 4 and 7 OVA aggregates generated at both pH 2.5 and 7.0 in their inguinal region and the delayed type hypersensitivity (DTH) response was assessed at various time points. The mice were footpad tested to determine their DTH reactions to native as well as various OVA aggregate forms. DTH reactions were elicited by the injection of various OVA aggregates (50 μg) as well as native OVA in the right footpad of each mouse and PBS into the left footpad. The thickness of each footpad was measured just before and after 36 h post-injection of OVA aggregates and PBS using a digital gauge caliper (Aero-space and Engineering Tools Ltd., Bolton, UK). DTH reactions were evaluated by the increase in footpad thickness as determined by the following formula (right footpad at testing time − right footpad at 0 h): (left footpad at testing time − left footpad at 0 h).

Determination of NO Production—Mice were sacrificed 1 week post booster, and peritoneal macrophages were isolated. The peritoneal macrophages were grown in culture plates and pulsed with OVA (final concentration 10 μg/well). After 24 h, 100 μl of culture supernatant was collected from each well, mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% H3PO4), and further incubated for 10 min at 25 °C and absorbance was determined at 550 nm in an ELISA reader (Bio-Rad).

T Lymphocyte Isolation from Spleens of Immunized Mice—Mice immunized with native as well as various aggregated forms of OVA were sacrificed on post day 5 after the last booster. T lymphocytes were isolated from the spleens of sacrificed mice as described elsewhere (34). Briefly, spleens isolated from animals belonging to various immunized groups were macerated and suspension was treated with ACK lysis buffer (0.15 mol/liter of ammonium chloride, 10 mmol/liter of potassium/bicarbonate, and 88 mmol/liter of edetic acid) for lysis of the red blood cells. The cell suspension was centrifuged at 1500 × g for 5 min, and the cell pellet was washed with Hanks’ balanced salt solution 3 times and resuspended in RPMI 1640 medium containing 10% fetal calf serum and 0.1% antimycotic mixture.

T-cell Proliferation Assay for Lymphocytes Isolated from Immunized BALB/c Mice—Lymphocyte proliferation assay was performed as described elsewhere (34). Briefly, lymphocytes isolated from the spleens of mice belonging to various immunized groups were incubated in round bottomed 96-well plates (2 × 105 cells/well) in 200 μl of RPMI 1640 medium with 10% fetal calf serum. Splenic cells isolated from groups of mice immunized with native or appropriate OVA aggregates were incubated with 20 μg of the corresponding matching forms of OVA. After 72 h, the cultures were pulsed with 0.5 μCi of [3H]thymidine. After 16 h, the plates were harvested onto glassfiber filter mats by the use of a Tomtec- Harvester-96 (Tomtec). The incorporated radioactivity was measured with liquid scintillation spectroscopy (Wallac-1450 Microbeta Trilux; PerkinElmer Life Sciences).

Lymphocyte Proliferation Assay for TCR-transgenic OT-I and OT-II Cells—OVA-specific T cell proliferation assay for TCR-transgenic OT-I and OT-II cells was performed by measuring [3H]thymidine incorporation, for determining antigen-specific response to different OVA aggregates. The assays were performed in Immunobiology Laboratory-2 at the National Institute of Immunology, New Delhi. Briefly, OT-I and OT-II splenocytes were isolated and cultured in 96-well culture plates at 1 × 105 splenocytes/well in 200 μl of complete RPMI medium. The splenocytes were stimulated with titrating concentrations of OVA aggregates. The plates were incubated in a humid atmosphere of 5% CO2 at 37 °C and pulsed after 60 h with 0.5 μCi/well for measurement of [3H]thymidine incorporation at the end of 72 h. Cells were harvested using a Filler-Mate cell harvester (PerkinElmer Life Sciences) and incorporation of [3H]thymidine was determined by a Top Count scintillation counter (PerkinElmer Life Sciences).

Cytokine Assay: Determination of IFN-γ, IL-4, and IL-12 by Sandwich ELISA—Th1 as well as Th2 cytokines induced by lymphocytes upon their culture in the co-presence of various aggregates of OVA were estimated using appropriate and specific biotinylated antibody pairs according to the manufacturer’s protocols. Briefly, 96-well microtiter plates were coated with 50 μl of the purified capture antibodies in carbonate/bicarbonate buffer (pH 9.5) at 4 °C. After the usual washing and blocking steps, 50 μl of the supernatant (isolated from cultured splenocytes after 48 h) was poured in each well for determining the level of cytokine induced. Plates were washed and incubated with biotinylated polyclonal goat anti-mouse cytokine detection antibody. Furthermore, after washing the plates, 100 μl of streptavidin-horseradish peroxidase conjugate was added to each well and plates were incubated for 30 min at room temperature. The plates were then washed and the colored complex was developed with tetramethylbenzidine. The absorbance was read at 450 nm with a microtiter plate reader (Bio-Rad). A known specific recombinant cytokine was used as standard for calculating the level of the given cytokine in the samples tested, and the concentration was expressed as pg/ml.

Dot Blot Assay—The native form of OVA (10 μg), various aggregate forms, and OVA released from various aggregates were spotted onto PVDF strips (with dimension of ~10 by 4
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Continuous Shaking over an Extended Time Period Induces Aggregation of Soluble OVA—Fluorescence intensity at 350 nm is a useful tool to detect aggregation in proteins (36). Taking this fact into consideration, the extent of light scattering was measured in OVA samples obtained after various incubation times (day 0 to 15) when OVA solution in PBS (pH 7.0) or glycine-HCl (pH 2.5) was shaken at 90 rpm. With an increase in incubation time, an enhancement in fluorescence intensity was observed in samples at both pH 2.5 as well as pH 7.0. The increase in fluorescence intensity for pH 2.5 samples was more prominent and significant when compared with samples incubated at pH 7.0. When compared with native OVA, fluorescence intensity was found to increase consistently with incubation times from 1 to 36 h suggesting aggregate formation. However, the magnitude of increment was less than 5-fold (data not shown). As shown in Fig. 1A, a, all the samples collected (at both pH 2.5 and 7.0) after various time periods exhibited a more than 7-fold increase in fluorescence intensity in comparison to native OVA in a time-dependent manner (up to day 15). The turbidity measurements determined at 350 nm for different forms of OVA obtained at various time periods also revealed a pattern in concordance with fluorescence intensity measurements thus offering further evidence in support of aggregate formation (Fig. 1A, b).

Extended Incubation with Shaking Leads to the Formation of β-Sheet-rich OVA Aggregates—The OVA samples obtained after various incubation periods were assessed for the presence of β-sheets employing Congo Red and ThT binding assays as well as CD spectroscopy. Congo Red, an amyloid binding dye, is used as a tool to monitor the formation of protein aggregates. The β-sheet conformation of amyloids is crucial for Congo Red binding. Congo Red exhibits a red shift in the absorbance spectrum upon binding to the amyloids (7). In the present study, Congo Red was incubated with aggregates obtained at various time points. The aggregates formed at longer incubation periods showed an increase in absorbance along with a slight red shift. Interestingly, a prominent red shift was observed in aggregates obtained upon incubation of protein for 15 days at pH 2.5 indicating conversion of all the secondary structures to β-sheet conformation. Protein aggregates formed at pH 7.0 did exhibit augmentation of absorbance at increasing incubations but the red shift in wavelength was lower compared with samples incubated at pH 2.5 (Fig. 1B).

ThT, another dye, exhibits enhanced fluorescence emissions upon binding to β-sheet-rich structures like amyloid aggregates of both fibrillar or non-fibrillar nature (8). The aggregates obtained at various time points were characterized employing the ThT binding assay. In general, the aggregates obtained at increasing incubation periods were found to bind ThT more explicitly (Fig. 1C). The aggregates obtained for pH 2.5 samples exhibited significantly enhanced bound fluorescence as compared with the corresponding aggregates obtained at pH 7.0. Interestingly, aggregate generated at pH 7.0 at the same incubation time period showed only 50% fluorescence of that observed for the pH 2.5 sample. The data suggest the formation of β-sheet-rich aggregates for both pH 2.5 and 7.0. However, the greater ThT binding exhibited by aggregated protein generated at pH 2.5 compared with pH 7.0 samples reflects that the fibrillar form for pH 2.5 aggregates to possibly appear earlier than the corresponding pH 7.0 aggregates (Fig. 1C). ThT binding with OVA aggregates was also observed using fluorescence microscopy. In concordance with ThT fluorescence spectral studies, fluorescence microscopy also revealed a similar pattern, i.e. aggregates obtained at longer incubations (both at acidic as well as neutral pH) exhibited increased fluorescence (Fig. 2A). ThT and Congo Red binding assays suggest that samples agitated for longer periods (either at pH 2.5 or at 7.0) harbor more β-rich structures than aggregates obtained at shorter incubations.

The far-UV CD spectrum of a protein is highly sensitive to its conformation. In fact, it is especially useful for offering
insight regarding the secondary structure of a protein. Fig. 2B highlights shaking induced conformational changes in the structure of OVA. Native OVA exhibited two minima, one at 208 nm and another at 222 nm, which is indicative of a $\alpha$-helical structure. With an increase in shaking time, the negative peak at 208 nm began to disappear, whereas the peak at 222 nm started shifting toward the left to lie between 215 and 222 nm, which indicates loss of the $\alpha$-helical structure with the appearance of $\beta$-sheets. The aggregates formed at both acidic as well as neutral pH conditions exhibited two negative peaks although the negative ellipticity was decreased in comparison to native OVA. A marked difference for pH 2.5 and 7.0 samples was observed upon further incubations. The aggregate obtained on day 4 (pH 2.5) exhibited a significant loss in negative ellipticity at 208 nm and the minimum at 222 nm shifted so as to lie between 222 and 215 nm. On the other hand, the protein aggregate generated at the same time period upon incubation at pH 7.0 did not exhibit a significant change when compared with the pH 2.5 sample (Fig. 2B). Day 7 sample, formed upon incubation at pH 7.0, exhibited a CD spectrum with a different pattern than its pH 7.0 counterpart, which matched with the spectrum obtained for the day 10 aggregate formed at pH 7.0. These aggregates exhibited a much higher loss in negative ellipticity at 208 nm than the aggregates formed earlier and also showed a shift in the 222 nm peak toward 215 nm. Similar results were obtained for

FIGURE 1. Continuous agitation for extended time periods induces aggregation of soluble OVA. A, Rayleigh scattering measurements at 350 nm for aggregates formed at various time points in both acidic and neutral ambiances (a). Turbidity measurements for OVA aggregates formed at various time points at pH 2.5 and 7.0 (b). Error bars exhibit standard error of three independent experiments. B, steady state absorption spectra of Congo Red bound to various aggregates obtained at pH 2.5 (a) and 7.0 (b). The aggregates were scanned in the 400–700 nm range. C, ThT binding assay of various OVA aggregates formed at pH 2.5 (a) and 7.0 (b). ThT emission spectra were obtained by excitation at 450 nm and emission in the range of 460–560 nm. Data are representative of at least three independent experiments with similar observations.
day 15 aggregates formed at both pH 2.5 and 7.0 with almost complete loss in 208 nm minima and a major shift toward 215 nm indicating the presence of only β-sheets, a structural feature of fibrillar amyloids (Fig. 2B).

Aggregates Formed at pH 7.0 Have Fibrillar whereas Aggregate Species Generated at pH 2.5 Have Suprafibrillar Morphology—The morphology of aggregates formed after various incubation periods at various pH conditions was elucidated employing electron microscopy. As shown in Fig. 3, aggregates obtained upon shaking at different pH conditions acquired varied morphologies after the same incubation periods. Fibrillar structures resembling typical amyloid fibrils appeared on day 10 postincubation for OVA incubated at pH 2.5 (Fig. 3A), whereas incubation at pH 7.0 ensued in amyloid fibril formation only after 15 days of incubation. In contrast, incubation of OVA at pH 2.5 revealed a branched suprafibrillar structure on day 15 postincubation (Fig. 3A).

The Sturdy Fibrillar Amyloids Release OVA in a Sustained Manner—The release of precursor OVA from its aggregates (formed at pH 2.5 and 7.0) was monitored over an extended time period (Fig. 3B). The protein aggregates (formed at pH 2.5 or 7.0) are found to release OVA in a time-dependent manner. The protein aggregates generated at pH 7.0 exhibited better release than the corresponding pH 2.5 aggregates. Release kinetics of various aggregates was monitored for a total of 15 days (Fig. 3B). Aggregates generated on day 2 of incubation (at pH 2.5 and 7.0) showed a burst release for the initial 3 to 4 days that leveled off subsequently. The aggregates formed at day 4 (for both pH 2.5 and 7.0) were found to release the precursor OVA in a steady manner that peaked at day 12 (for pH 2.5) and 10 (for pH 7.0) and plateaued at day 15. Aggregates generated upon 7 days of incubation exhibited varied release kinetics, the one formed at pH 2.5 could only feebly release OVA in contrast to the day 7 aggregate generated at pH 7.0, which exhibited a

FIGURE 2. Extended shaking of soluble OVA leads to formation of β-sheet-rich aggregates. A, ThT binding to various OVA aggregates (100 μg) as revealed by fluorescence microscopy. ThT (30 μM) was incubated with OVA aggregates for 30 min; 5 μl of the suspension was placed onto the glass slide and observed under fluorescence microscope. B, secondary structure determination of various OVA aggregates by far UV-CD. Native OVA and various aggregates (200 μg/ml) were scanned in the range of 200–250 nm. Data are representative of at least three independent experiments with similar observations.
slow and sustained release that reached plateau after 13 days. The aggregates obtained upon 10 and 15 days of incubation at pH 2.5 or 7.0 were also observed to release OVA. Hence, both fibrillar as well as suprafibrillar forms of OVA were found to release the protein.

With an increase in the protein concentration in a given sample, its intrinsic fluorescence increases because of the increased number of Trp and Tyr residues. Therefore, we also monitored the intrinsic fluorescence of the protein released in the surrounding milieu (PB, pH 7.4). The intrinsic fluorescence profile

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**FIGURE 3.** A, fibril maturation as revealed by transmission electron microscopy. Various aggregates (100 μg) were coated onto the grid. Images shown are those selected after similar observation of at least two independent experiments performed for each sample. B, kinetics of OVA release from various OVA aggregates. In vitro release kinetics of OVA from various OVA aggregates obtained at pH 2.5 (a) and 7.0 (b) for a period of 15 days as measured by determining absorbance at 280 nm. Kinetics of OVA release from various OVA aggregates generated at acidic (c) and neutral (d) pH as monitored by measuring intrinsic fluorescence with excitation at 280 nm and emission in the range of 300–400 nm. Data are representative of three independent experiments with similar observations.
of the released OVA for various aggregates was in agreement with those observed for spectrophotometric results. Intrinsic fluorescence of the materials released from day 15 aggregates formed either at pH 2.5 or 7.0 attained a substantial value suggesting release of OVA (Fig. 3 B, c and d).

Besides Monomer, the Aggregates Possibly Release Dimeric as Well as Oligomeric Forms of OVA—The elution profile of OVA released after 12 days of incubation from various aggregates is shown in Fig. 4 A. In almost all the fractions, peaks are observed in the void volume apart from another fully resolved and distinct peak corresponding to the released monomeric form of protein. The peaks corresponding to the void volume may be dimeric or multimeric forms of released OVA. This indicates that the released fraction of OVA from various aggregates is a mixture of monomeric, dimeric, as well as multimeric OVA (Fig. 4 A). Western blot analysis employing OVA-specific polyclonal and 3G2E1D9 monoclonal antibodies further supported the possible presence of various forms (monomeric/dimeric/oligomeric) of OVA released from aggregates (Fig. 4 B, a and b). Immunoblots stained with polyclonal anti-OVA antibody showed OVA release from day 7 aggregate formed at pH 7.0 after 4, 8, 12, and 15 days corresponding to lanes i, ii, iii, and iv, respectively (Fig. 4 B, a). Surprisingly, post 4 and 8 days of release, no monomeric band was observed, although bands of higher molecular weight that might be corresponding to dimer or higher oligomeric forms can be seen in Fig. 4 B, lanes i and ii. After 12 and 15 days of release, a distinct monomeric band in addition to higher molecular weight proteins can be observed in Fig. 4 B, lanes iii and iv. Similarly, Western blot performed using anti-OVA 3G2E1D9 monoclonal antibody shows concordig
result with that obtained for polyclonal antibody (Fig. 4B, b). Lanes i, ii, and iii correspond to OVA released after 8, 12, and 15 days, respectively, from day 7 aggregate formed at pH 7. Monomeric as well as higher oligomeric forms of OVA are seen to be present in the released materials after 12 days of release.

The Released OVA Seems to Acquire a Native Configuration and Does Not Exhibit General Characteristics of Amyloidal Aggregates—A fixed amount of released OVA from various aggregates (generated at pH 2.5 and 7.0) was analyzed for light scattering at 350 nm. As shown in Fig. 4B, c, the protein materials released from various aggregates exhibited light scattering very similar to that of native OVA. Moreover, the released OVA from various aggregates also showed binding to ThT more or less very similar to native OVA (Fig. 4B, d).

Antibodies Developed Upon Administration of Aggregated Forms of OVA Can Recognize Native Antigen—The immune response evoked in the host upon immunization with various aggregate forms of OVA was assessed on the basis of ELISA and dot blot assay. First, wells of ELISA plates were coated with various forms of OVA aggregates and were allowed to react with antibodies generated upon immunization with the corresponding aggregate forms. OVA fibrils formed either at pH 2.5 or 7.0 were found to react explicitly with the generated antibodies. Antibodies developed against aggregates formed either at pH 2.5 or 7.0 exhibited very similar reactivities. Dot blot assay results supported the ELISA findings that antibodies reactive to various OVA aggregate forms are formed upon their immunization (Fig. 5A).

Subsequently, the potential of the antibodies to recognize the native form of OVA was assessed. For ELISA, wells of microtiter plates were coated with native OVA and evaluated for its reactivity with antibodies developed against both native as well as various other forms of OVA aggregates. ELISA analysis revealed that antibodies generated against various forms of aggregates (formed both at pH 2.5 or 7.0) could recognize the native form of OVA (Fig. 5B, a and b). Aggregates generated on day 10 as well as day 15 (at both pH 2.5 and 7.0) were also successful in generation of anti-native OVA antibodies. Interestingly, day 4 aggregate for pH 2.5 as well as days 4 and 7 aggregates for pH 7.0 exhibited better antibody production than the native OVA itself. The specificity of the generated antibodies was further ascertained by dot blot assay. To perform dot blot, native OVA was coated on PVDF strips and its reactivity was checked for antibodies generated against various forms of OVA aggregates (both for pH 2.5 and 7.0 samples) formed at various time points. The antibodies developed upon immunization with both non-fibrillar as well as fibrillar OVA aggregates were found to recognize native OVA (Fig. 5B, c).

OVA Released by Various Forms of OVA Aggregates Interacts with Anti-native OVA Antibodies of Monoclonal as Well as Polyclonal Origin—The incubation of fibrils as well as other forms of OVA resulted in the release of precursor OVA into the surrounding milieu. We wondered whether various in vitro released species of OVA are reactive to native OVA-specific antibodies (of polyclonal as well as monoclonal origin); to ensure this we performed indirect ELISA and a dot blot assay. For ELISA, we coated the wells with OVA (2 μg/well) released in vitro from each aggregate (both non-fibrillar or fibrillar) and allowed it to interact with polyclonal anti-native OVA antibodies (which may recognize mature aggregates/fibrils as well). Proteins released from various aggregates generated either at pH 2.5 or 7.0 were found to react with anti-native OVA antibodies (Fig. 5C, a and b). Next, we performed a dot blot assay by coating the released OVA onto PVDF strips and checked their reactivity with antibodies raised against native OVA as done for ELISA. In concordance with ELISA data, the anti-native OVA antibodies recognized the OVA released from various aggregates (Fig. 5C, c).

To work on our hypothesis that the released OVA might be attaining a native conformation, we exploited the 2D11 antibody, which is an anti-OVA mouse monoclonal antibody showing high affinity for OVA in its native form, but is not able to recognize denatured OVA. ELISA as well as dot blot results enumerate the recognition of released OVA from various aggregates with 2D11 antibody (Fig. 5D, a and b). Native OVA as well as OVA released from various aggregates exhibit practically similar reactivities with 2D11 antibody. Similarly, dot blot data also reveals that 2D11 antibodies recognize both mono as well as oligomeric forms of released OVA. However, 2D11 antibodies fail to recognize the mature aggregates/fibril form of proteins (Fig. 5D, c).

Macrophages Phagocytose OVA Aggregates—The sustained release kinetics observed for various OVA aggregates persuaded us to analyze if they can be exploited as vaccine candidates. Because aggregates obtained at day 4 and 7 for pH 2.5 and 7.0, respectively, were found to release OVA in a slow and sustained manner. Therefore, we investigated their uptake by macrophages, which are professional antigen presenting cells. As shown in Fig. 6a, a punctuate fluorescence (co-localization with endo-lysosomal compartment) along with a diffuse pattern localized throughout the intracellular compartment of the macrophages is observed.

The OVA Aggregate-based Immunization Evokes Th1 Cytokines in the Host—Th1/Th2 bias was assessed in animals immunized with day 4 and 7 aggregates (formed at pH 7.0 and 2.5). The levels of Th1 cytokines, IL-12 and IFN-γ, were found to be significantly elevated in animals immunized with day 4 (formed at pH 7.0 and pH 2.5) and day 7 (formed at pH 7.0) aggregates in comparison to native OVA (p < 0.01) (Fig. 6, b and c). Although day 7 aggregates formed at pH 2.5 led to the generation of higher levels of Th1 cytokines, the increase was only moderate as compared with native OVA (p < 0.05). On the other hand, insignificant levels of Th2 cytokine (IL-4) were induced in the groups immunized with day 4 (generated at pH 7.0 and 2.5) and day 7 aggregates (formed at pH 7.0) (Fig. 6d). The day 7 aggregate (generated at pH 2.5) also exhibited generation of moderately lower levels of IL-4 as compared with native OVA, which showed elevated levels of IL-4 in comparison to control (Fig. 6d).

We also determined antibody isotype switching by evaluating the OVA aggregate-specific IgG1 and IgG2a response in the serum of immunized animals. Animals immunized with day 4 (formed at pH 2.5 and 7.0) and day 7 (generated at pH 7.0) aggregates showed a significantly higher IgG2a/IgG1 ratio as compared with native OVA (p < 0.01). On the other hand, day 7 aggregates formed at pH 2.5 showed only a moderate increase
FIGURE 5. A, induction of aggregate-specific antibodies upon immunization with OVA aggregates. OVA aggregates formed at pH 2.5 (a) and 7.0 (b) were allowed to interact with aggregate-specific antibodies employing ELISA. In the next set of experiments, various amyloid aggregates were coated onto PVDF strips and investigated for binding with antibodies generated upon immunization with corresponding aggregates (c). B, OVA aggregate immunization also induces native OVA-specific antibodies. The ELISA plate was coated with native OVA antigen as described under “Experimental Procedures” and reacted with sera obtained from the animals immunized with various aggregates formed at pH 2.5 (a) and 7.0 (b). Native antigen coated onto the PVDF membrane reacted with antibodies produced in response to immunization with various aggregates as revealed by dot blot assay (c). C, OVA released from various aggregates interacts with native OVA-specific antibodies. ELISA plate was coated with OVA released from aggregates formed at pH 2.5 (a) and 7.0 (b) and allowed to react with polyclonal antibodies produced in response to immunization with native OVA. For the dot blot assay, OVA released from various amyloid aggregates were coated onto PVDF strips and evaluated for reactivity with anti-native OVA polyclonal antibodies (c). D, OVA released from various aggregates reacts with 2D11 anti-OVA monoclonal antibody that recognizes only native but not denatured OVA. ELISA plate was coated with OVA released from aggregates formed at pH 2.5 (a) and 7.0 (b) and allowed to react with 2D11 anti-native OVA monoclonal antibodies. OVA released from various amyloid aggregates and amyloid aggregates themselves were coated onto PVDF strips and evaluated for reactivity with anti-native OVA 2D11 monoclonal antibody by dot blot assay (c). 2D11 antibody recognized OVA released from various aggregates as well as native OVA but failed to react with aggregates themselves. For control, a nonspecific protein (human serum albumin) was coated onto the membrane and allowed to react with the respective antibody. OVA-R denotes OVA released from various aggregates. Data are representative of at least three independent experiments carried out for each sample.
in the IgG2a/IgG1 ratio in comparison to native OVA ($p < 0.05$) (Fig. 6e).

T-cell Proliferative Response in Lymphocytes Isolated from Immunized BALB Mice—Lymphocyte proliferation in response to immunization with a prospective candidate vaccine is used as a parameter to assess its vaccine potential. Lymphocytes isolated after 1 week post-booster from the spleens of mice immunized with native OVA as well as day 4 and 7 aggregates formed at both pH 2.5 and 7.0 exhibited proliferation upon their exposure to native OVA (Fig. 6f). The proliferative response of lymphocytes obtained from animals immunized with day 4 (formed at pH 2.5 and 7.0) and day 7 (formed at pH 7.0) aggregates was significantly higher when compared with native OVA ($p < 0.01$), however, day 7 aggregate formed at pH 2.5 showed a
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OVA Aggregates Evoke Delayed Type Hypersensitivity in Immunized Animals—To evaluate the ability of various OVA aggregates to induce a cell-mediated immune response, we immunized mice subcutaneously in the lower abdominal region. Native OVA was used as immunogen to determine whether they need an adjuvant for evoking a desirable immune response. Seven days after immunization, mice were exposed to native OVA or PBS via the footpad and footpad swelling was measured at various time intervals. Immunization with day 4 (generated at pH 2.5 and 7.0) and day 7 aggregates formed at pH 7.0 resulted in the generation of a strong DTH response ($p < 0.01$), whereas the day 7 aggregate formed at pH 2.5 resulted in a generation of a moderate DTH response ($p < 0.05$) as compared with native OVA (Fig. 6g).

Nitric Oxide Production—Formation of NO is used as an index of the activated macrophage population. One week post booster, generation of NO was evaluated in macrophages isolated from mice immunized with native OVA and day 4 as well as day 7 OVA aggregates. Maximum NO production was observed in macrophages isolated from mice given day 7 aggregate formed at pH 7.0 after 24 h of pulsation ($p < 0.01$), followed by day 4 aggregate generated at pH 2.5 and day 4 aggregate formed at pH 7.0. An augmented NO production in comparison to native OVA was also observed in day 7 aggregate formed at pH 2.5 ($p < 0.05$) but was not as significant as observed for other aggregates (Fig. 6h).

Lymphocyte Proliferative Response in Splenocytes Isolated from TCR-transgenic OT-I and OT-II Mice—To obtain a clear picture about the humoral and cellular immune responses evoked, we also analyzed lymphocyte proliferative response in splenocytes isolated from TCR-transgenic OT-I and OT-II mice that exhibited T cells with TCR specific for OVA Class I and OVA Class II peptides, respectively. Splenocytes isolated from TCR-transgenic OT-I and OT-II mice were treated with native OVA as well as day 4 and 7 OVA aggregates formed at pH 2.5 and 7.0, respectively. As shown in Fig. 7, the proliferative response of OT-I and OT-II splenocytes with day 4 (formed at pH 2.5) and day 7 (formed at pH 7.0) aggregates was significantly higher when compared with native OVA ($p < 0.05$). Day 7 OVA aggregate formed at pH 7.0 also showed a slightly higher proliferative response at a fixed dose of OVA (20 μg) at the 1 week post booster time point.

T cell proliferation in comparison to day 4 OVA aggregate formed at pH 2.5.

DISCUSSION

Amyloid fibrils belong to the group of self-assembled ordered nanostructures generated upon improper folding of polypeptides/proteins (2). Although pathogenesis of several human diseases is linked to amyloids, growing evidence indicates that amyloids might be actively participating in several biological events. A few recent reports have elaborated that in vitro synthesized amyloids when administered into animals elicit biological functions of native protein (23, 24). Maji et al. (23) found that long-acting gonadotrophin releasing hormone analogs are

FIGURE 6. OVA aggregates are taken up by professional antigen presenting cells and also evoke Th1 biased immune response in the immunized animals. a, green fluorescence shows the uptake of day 4 aggregate obtained at pH 2.5 (upper panel) and day 7 aggregate obtained at pH 7.0 (lower panel) by macrophages isolated from thioglycollate-primed BALB/c mice. Data are representative of at least three independent experiments carried out for each sample. OVA aggregates (day 4 and 7 aggregates formed both at pH 2.5 and 7.0) mediated Th1/Th2 bias was ascertained by determining the cytokine response in splenocyte culture supernatant belonging to various immunized groups following the protocol described under "Experimental Procedures," i.e., IL-12 (b), IFN-γ (c), and IL-4 (d). To further confirm the Th1/Th2 polarization upon immunization with day 4 and 7 aggregates (generated at pH 2.5 and 7.0), the sera of immunized animals were analyzed for the presence of IgG2a and IgG1 isotype (e). Moreover, OVA aggregates induce strong DTH response, lymphocyte proliferation, and enhanced NO production as revealed by $F_{\text{th}}$, respectively in BALB/c mice. The DTH response was evaluated by measuring footpad swelling in mice immunized with day 4 aggregate, day 7 aggregate, native OVA, and PBS. The lymphocyte proliferation assay was performed to measure lymphocyte proliferation of OVA-specific T lymphocytes. Lymphocytes isolated from various immunized groups were stimulated with a corresponding set of matching formulations of OVA. The cells were allowed to grow in the presence of [3H]thymidine as described under "Experimental Procedures." The incorporation of labeled nucleotide during DNA synthesis of proliferating cells was measured and denoted as cpm. NO was determined in macrophages isolated from mice immunized with various aggregates. The data represent mean of three determinants ± S.D. and are representative of at least three independent experiments with similar observations. Various immunized groups were compared to determine the statistical significance of the data using Student's t test analysis with $p < 0.05$ level of significance. Day 7 aggregate formed at pH 7.0 versus native OVA, $p < 0.01$; day 4 aggregate formed at pH 7.0 versus native OVA, $p < 0.01$; day 7 aggregate formed at pH 2.5 versus native OVA, $p < 0.05$; day 4 aggregate formed at pH 2.5 versus native OVA, $p < 0.01$.
able to form amyloids \textit{in vitro}, which can sustain the release of monomeric drug, the therapeutic molecule, both \textit{in vitro} and \textit{in vivo}. Similarly, a controlled release of insulin monomers from \textit{in vitro} synthesized pre-fibrillar oligomers made from insulin has also been reported (24), demonstrating them to be a potential therapeutic agent of long-acting peptide drugs. The investigators propose that amyloid fibril or pre-fibril forms can act as depots and possibly release the bioactive form of the peptide/protein in a sustained manner. The bioactivity of the released peptide is realized from the fact that it binds to the specific receptors (\textit{c.f.} gonadotrophin releasing hormone receptor and insulin receptor) for which obviously a perfectly fitting conformation is required to activate the downstream signaling cascade. In these reports, it has been proposed that the amyloid form of protein may release the bioactive peptide retaining the conformational properties of the native state of the peptide. In the present study, in-house synthesized amyloid fibrillar and non-fibrillar aggregates of OVA were injected into mice and the antibodies raised against them were evaluated for their specificity with native form of OVA.

Morphological alterations in amyloids formed from the same protein under differing incubation ambiances are among the most intriguing aspects of amyloid attributes (37). The pH of the surrounding medium has been observed to be one of the most influencing factors on the characteristic morphology and properties of the resulting amyloid fibrils. It has been found that by manipulating pH conditions, whereas keeping other extrinsic factors constant, fibrils of various shapes and sizes of the same protein can be generated (24). In the present study, buffers with three different pH, glycine-HCl (pH 2.5), glycine-NaOH (pH 10), and PBS (pH 7.0), were used to induce OVA amyloid formation. Although pH 2.5 and 7.0 buffers could induce aggregation upon shaking, pH 10 buffer failed to induce aggregation (data not shown). Acidic ambience was found to induce better protein aggregation than neutral pH conditions (Fig. 1). Interestingly, the amyloidal aggregates for OVA obtained at two different buffering conditions exhibited altered properties with respect to content of $\beta$-sheet-rich structures and also to the morphological characteristics of formed aggregates (Figs. 1–3).

Moreover, the data presented in Figs. 1–3, in general, clearly reveal that pH 2.5 induces early fibril formation as compared with pH 7.0. This is particularly well demonstrated in Fig. 3A where the day 15 aggregate formed at pH 2.5 shows hugely branched suprafibrillar assembly in contrast to the pH 7.0 sample revealing two fibrillar threads separately lying over the surface. This might be explained on the premise that at pH 2.5, earlier formed fibrils would further adhered laterally upon longer incubation conditions that eventually led to the formation of suprastructures. The data also suggest that amyloid formation involves a hierarchial process where precursors formed at the initial stages act as a template for the growth of mature fibers that subsequently interact laterally to generate suprafibrillar structures. A schematic representation of the hierarchical process of fibril formation is outlined in Fig. 8. The two pH conditions, i.e. neutral versus acidic, seem to induce $\alpha \rightarrow \beta$ transitions in the protein following the same sojourn, however, this transition is more prompt at acidic conditions when compared with neutral pH conditions. It seems that the variations in the electrostatic repulsions or attractions in response to differing

![Schematic illustration depicting kinetics of fibril formation and potential of OVA aggregates to induce generation of antibodies that have ability to recognize both native as well as fibrillar OVA. The model depicts that formation of fibril from monomeric native OVA encompasses various intermediates such as abnormally folded monomers and oligomers. More specifically, it highlights that fibril immunization leads to generation of antibodies reactive to native as well as fibrillar OVA.](image-url)
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pH conditions may lead to formation of aggregates of varied morphologies. This implicitly suggests that the amyloid fibrils may have the potential to exhibit distinct forms depending on the incubation conditions.

Once it was ascertained that both acidic and neutral pH conditions induce the formation of amyloidial aggregates and fibrils of OVA with differing compactness and morphologies, we set about to study their tendency to release OVA, the precursor protein. The day 4 and 7 aggregates formed either at pH 2.5 or 7.0 were found to release OVA over a period of 10–15 days in a sustained manner. Beyond this period, OVA release from the aggregates attained equilibrium. Interestingly, the fibrillar aggregates formed at days 10 and 15 could also release OVA although the release was not much pronounced when compared with aggregates obtained at the day 4 and 7 incubation periods (Fig. 3B). To analyze the state of OVA released from various aggregates, size exclusion chromatography and Western blot analyzes of released OVA from various aggregates were performed. As seen in the size exclusion chromatography analysis (Fig. 4A), the released OVA from various aggregates seems to be in the monomeric, dimeric, as well as multimeric states although the monomeric OVA seems to be a dominant species in the mixture for all the samples. As can be seen in Western blot analysis performed using 3G2E1D9 monoclonal as well as polyclonal anti-OVA antibodies, the appearance of monomeric as well as oligomeric forms of the OVA can be detected by day 12 postincubation (Fig. 4B, a and b). Until day 8, bands corresponding to higher oligomeric forms of OVA can be seen but no monomeric band is observed. Immunoblot studies appear to indicate that release of OVA from various aggregated forms seem to follow a multistep kinetics. It is to be noted that the OVA dimers/oligomers that are released from the aggregates are SDS-insoluble, hence, did not run as a monomer in a reducing SDS gel, which is in concordance with several reports enumerating the formation of SDS-insoluble amyloid fibrils (38–41). This supports the view that the respective species are actually released from amyloid fibrils.

Furthermore, antigen antibody interactions revealed that antibodies generated upon immunization with various aggregates obtained at pH 2.5 or 7.0 could recognize native OVA in addition to aggregate forms (Fig. 5, A and B). A schematic illustration of the production of antibodies capable of recognizing fibrillar as well as native OVA upon fibril immunization is shown in Fig. 8. Induction of antibodies reactive to native OVA upon immunization with various aggregates suggests that either the fibrils themselves carry native antigen epitopes or the released OVA might be retaining the native epitopes, also both possibilities may happen simultaneously. Interestingly, day 4 aggregate formed at pH 2.5 and both day 4 as well as day 7 aggregates obtained at pH 7.0 exhibited elevated antibody production against native OVA than the sera obtained upon immunization with native antigen (Fig. 5B, a and b). The observed interaction of OVA with specific polyclonal antibody could be explained on the premise that aggregates may have acted as depots for the slow and sustained release of the antigen, which boosted immune cells over the time to generate antigen-specific antibodies. Moreover, OVA released under simulated conditions in vitro (from each aggregate obtained at pH 2.5 and 7.0) was tested for its reactivity with antibodies generated against the native form of OVA. The anti-native OVA polyclonal antibodies recognized the released OVA substantially, indicating possible retention of native epitopes in the released proteins (Fig. 5C). The observation was validated by employing 2D11 monoclonal anti-OVA antibody that specifically recognizes the native form of OVA but not the denatured form. Interestingly, anti-native OVA monoclonal 2D11 antibody recognizes native OVA as well as OVA released from various aggregates but not aggregates themselves (Fig. 5D). It indicates that the 2D11 antibody is specific to an epitope (conformational epitope) exposed in native OVA but inaccessible in aggregated forms. The reactivity of 2D11 antibody with native and released OVA but not to its aggregated form gives an indication that protein when released from aggregates may undergo a conformational change. The released precursor protein seemingly attains the conformation similar to that of native protein leading to the exposure of the epitopes accessible in native form, which, however, may be buried in the aggregated protein. The reactivity of two different OVA-specific monoclonal antibodies (3G2E1D9; capable of recognizing denatured OVA and 2D11; reactive to native OVA only not denatured form) used in the present study clearly demonstrates that OVA aggregates have the potential to release precursor proteins with intact linear as well as conformational epitopes. Biophysical characteristics such as light scattering and ThT binding of released OVA from various aggregates was also found to be very similar to that observed for native OVA (Fig. 4B, c and d). Moreover, the far UV CD spectra of the proteins released from various aggregates were also found to be similar to the spectrum obtained for native OVA (data not shown). Although these findings suggest that the released OVA possibly refolds to native protein and hence may harbor the conformational properties of the native form of the protein. However, it would be too premature to conclude that the proteins released from amyloids completely refold to the native protein conformation, as from one intact conformational epitope, we cannot declare conformational integrity of the overall structure.

The immune response data of the present study categorizes aggregate form of protein to act as a potential vaccine, whereby, slow release of antigen over extended time periods can elicit desirable immune responses without repeated booster requirements. Because the day 4 and 7 aggregates generated both at pH 2.5 and 7.0 exhibited slow and sustained release of OVA, we analyzed Th1/Th2 bias, lymphocyte proliferation, DTH response, and NO production in animals immunized with these aggregates. Day 4 and 7 aggregates (formed at pH 2.5 and 7.0) were found to induce significantly higher levels of Th1 cytokines as compared with native OVA (Fig. 6). Strong T cell proliferation and heightened DTH response also indicate a polarized bias for the cell-mediated response in animals immunized with OVA aggregates formed at pH 2.5 and 7.0, but not in those immunized with its native form. Moreover, a significantly higher amount of NO was produced by macrophages isolated from mice immunized with OVA aggregates (Fig. 6). Lymphocyte proliferative responses in OT-I and OT-II lymphocytes in response to stimulation with day 4 and 7 OVA aggregates obtained at pH 2.5 and 7.0, respectively (Fig. 7), are found in
concordance with the T cell proliferative responses obtained for lymphocytes isolated from immunized BALB/c mice. This indicates that OVA aggregate forms are successful in evoking both humoral as well as cell-mediated immune responses in the host. As shown in Fig. 6, b–h, the most desirable response among various aggregates was observed for day 7 aggregate (formed at pH 7.0) followed by day 4 aggregate (formed at pH 2.5), which was further trailed by day 4 (formed at pH 7.0) and day 7 (formed at pH 2.5) aggregates. The pattern observed appears to be correlated to the release kinetics of OVA from various aggregates because the day 7 aggregate (formed at pH 7.0) exhibited the best release kinetics when compared with other aggregates. It was followed by day 4 aggregate (formed at pH 2.5), which was found to exhibit better release kinetics than day 4 (formed at pH 7.0) and day 7 (formed at pH 2.5) aggregates. The results are consistent with a previous report where it has been demonstrated that the generation of desirable humoral and cellular immune responses is a function of kinetics of antigen delivery (42). Demento et al. (42) have shown that OVA despite being a weak immunogen in free form could elicit a strong humoral and cellular immune response when delivered in poly(lactic-co-glycolic acid) nanoparticles. They ruled out the adjuvant properties of encapsulating material, specifically poly(lactic-co-glycolic acid) (a biodegradable and biocompatible polymer). Rather, their findings enumerate that the slow and sustained release of OVA from poly(lactic-co-glycolic acid) nanoparticles led to better humoral and cellular immune responses in contrast to liposomes, which exhibited a burst release of OVA. Interestingly, they also found that sustained antigen release plays a crucial role in shaping a long-lasting effector T cell memory response, which led to effective clearance of Listeria monocytogenes, an intracellular pathogen (42).

Besides specific affinity between the TCR-peptide-MHC interface, the phenotype of T lymphocytes generated against a given antigen is generally regulated by its dose, chemical composition, and also by factors such as differential expression of co-stimulatory molecules, presence of adjuvants, cytokine milieu etc., which play important roles in differential Th1/Th2 bias (43–45). Although the potential of OVA aggregates to induce a strong humoral response can have a direct correlation with a sustained release potential, however, a Th1 bias of various observed immunological responses remained intriguing. It seems that preferential uptake of OVA amyloids by macrophages (Fig. 6a) because of their particulate nature has some co-relationship with the Th1-biased response as in an earlier study, it has been reported that OVA linked to beads is presented via MHC class I molecules by macrophages approximately $10^4$ times more efficiently than soluble OVA (46).

Fig. 9A highlights various sequential and conformational B-cell epitopes present in OVA. The various sequential epitopes have been predicted by COBEPto (47), an algorithm dedicated to predicting continuous B-cell epitopes. The conformational B-cell epitopes have been predicted using CBTOPE prediction algorithm (48). The cell-mediated immunity exhibited by OVA amyloid aggregates can also be in part due to some of the primary sequence stretches that have a strong propensity to form amyloids and simultaneously bear T cell antigenic determinants.

Tanaka et al. (49) identified three high β-aggregation propensity regions in OVA using algorithms like TANGO, PASTA, and AGGRESCAN; 39IAMSLALAMYV43 (blue), 172MLVNAVIFK43 (red), and Phe364–Val365 (green). They further proved by in vitro studies that 38LAMVY43 (a stretch of 39IAMSLALAMYV43 sequence) has a very strong tendency to form amyloid fibrils. Interestingly, a sequence, 39AMVYLGAKDSTR40, has been attributed to be a T-cell epitope of OVA, which harbors 39AMVY43 (50). The high β-aggregation propensity regions and T-cell epitope of OVA are shown in Fig. 9B.

Our results support the hypothesis that amyloid immunization induces native protein-specific immune responses because of the release of precursor proteins from the amyloid depot, which may bear conformational properties of native protein. However, it cannot be ignored that native protein-specific antibodies may also be generated in response to various linear epitopes that remained intact in the fibrils during their formation from precursor proteins. In other words, antibodies specific for linear epitopes of the native protein may cross-react with the fibrillar form. Nevertheless, our purpose of exploiting amyloid as a vaccine candidate is fulfilled even if the host immune system favors the latter option. It has been reported for
several amyloidogenic proteins to induce the formation of antibodies capable of recognizing both the fibrils as well as the native protein. Antibody HMB50 against the amyloid-forming protein Pmel17 is an example, in that this antibody recognizes fibrils (51) and also efficiently immunoprecipitates newly synthesized native, not yet aggregated protein (52). Many fibril-reactive antibodies additionally recognize SDS-denatured protein. An example would be the Pmel17-specific antibody HMB45 (51). Moreover, aggregation of recombinant epoietin (EPO) has been shown to induce antibodies that bind to native EPO, leading to pure red cell aplasia in subjects (53, 54). It is to be noted that in all the above discussed reports, release of native protein from amyloids has not been studied; nevertheless, the generation of native protein-specific immune responses is well elucidated. Reckoning with these findings, even if we do not take into consideration the release of soluble native protein from the amyloid depot, the presence of intact fibril itself in the system may boost the immune system to elicit a desirable immune response. In fact, amyloids significantly withstand biological proteolytic degradation when compared with the native form of the protein (2, 23, 24), which ensues in extending the residence time of the antigen much longer in circulation. This “stability effect” could strongly enhance its potency as a vaccine even if we ignore release of any precursor protein from amyloid as it may still facilitate the generation of antibodies that may cross-recognize native protein.

CONCLUSIONS

Although the immunoprophylactic responses can always be manipulated by including desirable immunomodulators, the categorical immune response induced in the host upon administration of OVA aggregates suggests that the system can be used as vaccine without inclusion of external excipients. Taking immune activation features into consideration, the self-assembled amyloid bodies can be exploited as potential vaccine candidates against both extra as well as intracellular pathogens.

Acknowledgments—We are thankful to former and present coordinators, IBL, for allowing us to avail the facilities of Institute’s Central Instrumentation Facility and also acknowledge the University Sophisticated Instrumentation Facility (USIF) for the transmission electron microscopy micrographs. We are grateful to Dr. Satyajit Rath (National Institute of Immunology, New Delhi, India) for the generous gift of TCR-transgenic OT-I and OT-II mice and also allowing us to use his laboratory facilities for conducting the experiments on the same.

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