Potential, pitfalls, and prospects of food allergy diagnostics with recombinant allergens or synthetic sequential epitopes

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This article aims to critically review developments in food allergy diagnostics with regard to the verification of specific IgE antibodies and the identification of the responsible allergens. Results of IgE-binding tests with food extracts are hampered by cross-reactive proteins, low-quality test agents, or both. Specificity can be increased by defining adequate cutoff values, whereas sensitivity can be improved by using high-quality test agents. IgE-binding tests with purified allergens enabled reliable quantification of allergen-specific IgE titers, with higher levels found in individuals with food allergy compared with individuals without food allergy. However, the overlap in individual test reactivity between allergic and nonallergic subjects complicates interpretation. Recombinant allergens and synthetic sequential epitopes enabled detection of sensitization profiles, with IgE specific to several allergens and substructures now being suggested as markers of severity, persistence, or both. However, high-power quantitative studies with larger numbers of patients are required to confirm these markers. IgE-binding tests merely indicate sensitization, whereas the final proof of clinical relevance still relies on family/case history, physical examinations, and provocation tests. Novel technologies promise superior diagnostics. Microarray technology permits simultaneous measurement of multiple IgE reactivities regarding specificity, abundance, reactivity, or interaction. Improved functional tests might enable reliable estimation of the clinical relevance of IgE sensitizations at justifiable expenses. (J Allergy Clin Immunol 2008;121:1323-30.)

Key words: *Food hypersensitivity, component-resolved diagnosis, recombinant food allergen, sequential epitope, immunoblot inhibition, skin prick test, IgE quantification, sensitivity, specificity, cross-reactivity*

Abbreviations used
sIgE: Allergen-specific IgE antibody
SPT: Skin prick test

Epidemiologic studies based on food challenges indicate that 1% to 10.8% of the general population have immune-mediated nontoxic food hypersensitivity, which is the most common trigger of anaphylaxis in young age.[1,2] Food allergy includes IgE-mediated and non–IgE-mediated syndromes, where IgE-mediated manifestations are responsible for the majority of food-induced, immediate-type, immune-mediated hypersensitivity reactions.[3] The development of an IgE-mediated response to food requires a series of molecular and cellular interactions, which involve antigen-presenting cells, T lymphocytes, and B lymphocytes.[4,5]

Depending on the route of sensitization, food allergy is the result of either genuine reactivity to comestibles through the gastrointestinal tract (class I food allergens) or secondary sensitization to cross-reactive food allergens as a consequence of the initial reactivity to homologous pollen-related allergens (class II food allergens).[6,7] The majority of class I food allergens are heat stable and resistant to degradation or proteolytic digestion, whereas class II food allergens are usually easily degradable.[8] Stable class I food allergens have the potential to induce severe reactions, whereas easily degradable class II food allergens tend to induce milder reactions often limited to oral allergy symptoms.[6,8,9] Another characteristic of food allergens is the occurrence of sequential (linear), as well as conformational (discontinuous), IgE epitopes. Sequential epitopes have been suggested to be more important in class I food allergy, whereas conformational epitopes have been suggested to be more relevant in class II food allergy.[10]

Accurate diagnosis of food allergy and appropriate treatment options depend on the verification of functionally relevant, allergen-specific IgE antibodies (sIgEs), as well as on the identification of the responsible allergenic molecule or molecules. Today, a variety of *in vivo* and *in vitro* test systems are available to investigate sIgEs as biomarkers for allergy specification.[11] However, a positive sIgE test result merely identifies sensitization to a particular allergen and does not permit definitive differentiation between clinically relevant IgE reactivity (ie, reactivity that is capable to cross-link FcεRI receptors) and IgE reactivity not accompanied by clinical symptoms (ie, reactivity without an effector cell response).[11-13] As a consequence, the clinical interpretation of sIgE test results with food extracts is often impeded by clinically irrelevant food-food or pollen-food cross-reactive IgE antibodies, leading to positive test results in subjects without

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clinical food allergy.[14,15] A further problem is that commercially available food extracts are often not standardized, and the content of functional allergenic molecules varies based on the nature and quality of the food, the extraction procedure, and storage conditions.[16-20]

Double-blind, placebo-controlled food challenges overcome the problem of determining the clinical relevance of sIgE reactivity, which is why they are still the gold standard in food allergy diagnostics, against which all other approaches should be verified.[11,13,21] However, *in vivo* provocation tests with food allergens carry the risk of inducing severe allergic reactions.[11,22] Therefore functional sIgE tests to detect basophil activation *in vitro* by using either patients' cells directly or serum IgE coated to a basophil cell line have been suggested as surrogates.[23,24]

Progress in biochemistry and molecular biology allowed for the identification, cloning, and recombinant production of allergenic proteins, as well as the synthesis of IgE epitope–emulating peptides of a number of food allergens.[10,16,25] These new measures enabled component-resolved diagnostics of food allergy by detecting and quantifying IgE antibodies specific to a protein or even a sequential epitope. Component-resolved diagnosis revealed individual sensitization patterns to (1) different proteins of an allergenic food, (2) homologous proteins in different foods, and (3) different epitopes of a single allergenic molecule.[26-31] Nevertheless, cumulative analyses of these results also revealed the presence of common sensitization patterns to major allergenic molecules of a single food, as well as to immunodominant IgE epitopes in a single allergenic molecule.[26,27,32,33] These findings encourage the use of panels of purified native or recombinant allergenic molecules and the use of synthetic sequential epitopes for an elaborate molecular analysis of sensitization patterns. This promises refined diagnosis, risk assessment, and prediction in food allergy. The present review aims at providing a general conspectus on the basis and current effect of diagnostic IgE-binding tests with recombinant food allergens or the respective sequential epitopes.

IMMUNOBLOT ANALYSES ALLOW FOR THE IDENTIFICATION OF TARGET MOLECULES

Immunoblot analysis of sIgE reactivity to food extracts with labeled anti-human IgE antibodies after gel electrophoresis and Western blotting of the allergenic protein source first enabled (1) the identification and discrimination of allergenic molecules from a single source and (2) the detection of individual sensitization patterns to specific allergenic molecules in different but cross-reactive sources.[34] In many cases overall sensitization to immunodominant proteins, as well as individual sensitization to minor allergenic proteins, was observed.[35-39] Nevertheless, the pattern of sIgE reactivity can be highly disparate among patients with allergy to the same food.[39,40]

Immunodominant proteins with identical or highly similar molecular masses were detected in extracts of different sources, indicating food-pollen[41] or food-food cross-reactivity.[35,36,42,43] Preabsorption of patient serum with purified native or recombinant allergenic molecules before immunoblot experiments confirmed the expression of cross-reactive sIgEs, which are capable of binding proteins from different sources with conserved binding sides and sufficient sequential, as well as structural, homology.[44] Accordingly, immunoblot inhibition studies demonstrated attenuation of sIgE reactivity to immunodominant proteins of crude allergen extracts, as well as to purified allergens, by means of preabsorption of the sera with purified proteins from a homologous source.[36,37,45-47] Reciprocal IgE antibody neutralization experiments verified the concept of pollinosis as a trigger of food allergy in terms of secondary cross-reactivity of pollen-specific IgEs to class II food allergens.[48] Accordingly, recombinant Bet v 1, the major birch pollen allergen, was shown to act as a potent inhibitor of IgE cross-reactivity to the homologous proteins from apple, carrot, celery, hazelnut, and peach,[45,47] whereas homologous Api g 1, Mal d 1, and Dau c 1 from celery, apple, and carrot were inefficient in inhibiting IgE reactivity to Bet v 1.[45] This can be explained by the "polyclonal" situation of the immune response, in which a number of different IgE antibodies recognize the primary antigen but only a few of them react with the secondary antigen.

Immunoblot analyses are also useful in evaluating the allergen-specific influence on the course of food allergy (eg, regarding the effect of individual sIgE sensitization patterns to different allergenic molecules of a single source). As an example, immunoblot analyses elucidated why allergy to certain plant foods manifests with relatively mild clinical conditions in patients with birch pollen allergy from central Europe,[6,9,14] whereas the same comestibles have the capacity to provoke severe systemic reactions in patients without (birch) pollen allergy from the Mediterranean area.[6,49] It could be demonstrated that Bet v 1–homologous class II food allergens are responsible for immunodominant sIgE reactivity in patients with birch pollen allergy, whereas sera of patients without (birch) pollen allergy frequently react to class I food allergenic lipid transfer proteins of these comestibles.[28,50-52]

Taken together, immunoblot experiments are an essential basis for the development of *in vivo*, as well as *in vitro*, sIgE test systems with purified allergens. However, an immunoblot detection of sIgEs is not a proof of clinical relevance because a single IgE epitope is sufficient for the *in vitro* reactivity.

SKIN TESTS WITH PURIFIED ALLERGENIC MOLECULES

The easiest approach in establishing whether a patient possesses sIgEs is a skin prick test (SPT) with commercially prepared allergen extracts.[53] The diagnostic potential of skin tests depends first of all on the quality of the test agents. Unfortunately, quality and composition of the available, mostly nonstandardized food extracts is highly variable. One might speculate that this is especially true for labile plant food allergens. However, skin tests with fresh extracts or prick-by-prick approaches with fresh food frequently revealed higher assay sensitivity in comparison with tests with commercial extracts for all classes of food allergens.[54-57] Nevertheless, skin tests with commercial food extracts of class I food allergens are usually characterized by high sensitivity and negative predictive values of more than 90%, whereas their specificity is generally poor, and a positive test has, on average, a 50% positive predictive value.[58] In contrast, skin tests with commercial food extracts of class II food allergens can also be vitiated by low sensitivity (eg, because of the low proteolytic stability of the allergenic components).[28,59-61]

Just as with *in vitro* sIgE tests (see below), diagnostic skin tests are further hampered by the fact that positive test results are frequently seen in a considerable proportion of individuals without adverse reactions to the respective food.[54,62-66] The significance of a positive test result with a food extract is mainly deteriorated

by the presence of multiple molecules, each with the potential to cross-react with IgEs specific to homologous proteins of different biologic sources.[14,17,67] As a consequence, positive testing with a food extract frequently just reflects an initial sensitization to, for example, a homologous pollen allergen, where cross-reactivity is often not (yet) correlated with the development of clinical food allergy.[14] Thus, a negative skin test result is very likely to confirm the absence of IgE-mediated food hypersensitivity, whereas positive test results merely indicate sensitization.

Nevertheless, studies aimed at evaluating the diagnostic capacity of SPTs were capable of defining wheal size diameters that represent predictive decision points for clinical food allergy. However, the accuracy of SPT decision points depends on the quality of the test agent.[33,68-71] Purified native or recombinant allergenic molecules represent standardized test agents of precisely defined quality and promise crucial diagnostic progress. Moreover, recombinant allergenic molecules enable component-resolved identification of individual sensitization patterns and thus discrimination of different patient groups with respect to, for example, severity scores. However, only a few comparative SPT studies with purified food allergens have been conducted,[28,72-74] which is mainly due to the high demands of approval in clinical trials as requested by the institutional review boards and regulatory authorities to ensure patient safety (test allergens have to be licensed as biotechnologic products). These few studies verified a superior diagnostic potential of recombinant allergens. However, the sensitivity of a skin test with a single molecule is often lower compared with that of a test with an allergen extract that contains several different allergenic molecules. Hence, skin tests with recombinant molecules need to include panels of recombinant allergens covering all immunodominant structures present in a given food.[72,74]

QUANTIFICATION OF IgE ANTIBODIES

Today, sIgEs can be measured within the clinical routine by using commercially available assays (eg, RASTs and EASTs, respectively), ELISAs, and highly reliable quantitative and automated methods using the fluorescence enzyme immunoassay or the reverse sandwich immunoassay with direct chemiluminescent technology.[75,76] These *in vitro* assays are especially useful when SPTs cannot be performed or interpreted in patients with generalized dermatitis or in those who must continue to take antihistaminic medications.[77] A further advantage of *in vitro* sIgE determinations is the elimination of the risk for systemic reactions existing in all provocation tests.[78] Quantitative *in vitro* assays also offer advantages over the at-best-semiquantitative immunoblot analyses. The former enable investigation of allergens in their native form, whereas the denaturing conditions of the latter (SDS gel electrophoresis) include a stronger risk of not presenting native IgE-binding epitopes, possibly causing detection failures.[79,80]

RAST assays with crude allergen extracts allowed, for the first time, estimation of sIgE serum concentrations,[81-83] but the accuracy of *in vitro* sIgE determinations is highly variable.[54,84,85] This is, for example, due to lacking standardization of the test agents, as well as to instability of some of the respective allergenic proteins.[16-20] Moreover, some clinically relevant food allergens with high structural stability and resistance to the gastrointestinal environment demonstrate only low RAST signals because of their low abundance in the extracts.[86] In general, the universal problem in food allergy testing (ie, relatively high sensitivity with overall low specificity) also applies for quantitative sIgE determinations (see skin testing section).

IgEs specific to allergenic proteins are the causative agents in the clinical manifestation of food allergy. Therefore, one would expect a direct correlation between their titers and the probability/severity of the allergic symptoms. Indeed, on average, higher sIgE levels were found in subjects with food allergy compared with those in subjects without food allergy. Nevertheless, depending on the food investigated, a huge overlap in individual sIgE reactivity between healthy subjects and subjects with food allergy also became evident.[35,87-90] Several reasons might be suggested to explain the overlap in test reactivity. First, low sIgE signals in allergic patients might be attributed to sensitization to a low-abundance, highly resistant protein with particular allergenic potency. Second, allergen-specific IgGs with the capacity to compete for the IgE-binding epitopes might cause invalid effector cell activation, despite the presence of adequate sIgEs[91-93]; a few studies even hint at a high IgE/IgG ratio as a prognostic marker in food allergy,[94,95] whereas others did not verify such a correlation.[96] Because IgE versus IgG competition might also interfere with IgE quantifications, novel approaches initially capture all serum IgE at a solid phase and use labeled allergen for the detection of the captured antibodies.[97] Third, qualitative differences of the respective sIgEs might be responsible for uneven efficacy in effector cell activating.[98] Finally, monoclonality is sufficient for *in vitro* reactivity, whereas polyclonality is required for effector cell activation (see the introduction to this article).

In accordance with similar SPT attempts (see above), efforts were made to estimate cutoff values (decision points) of sIgE titers that enable prediction of food challenge outcome.[58,99,100] In general, these studies verified that increasing sIgE titers correlate with an increasing risk of reactivity on challenge and that reasonable threshold values can be defined to avoid challenges in a subgroup of patients.[58,71,89,101-105] However, diagnosis of food allergy based on decision points is hampered by a huge overlap in test reactivity between healthy subjects and patients with food allergy. The definition of more stringent cutoff values has the potential to improve the specificity, as well as the positive predictive value. However, this will inherently also result in a significant decrease of sensitivity, as well as in a reduced negative predictive value, by increasing the number of false-negative results. Moreover, the predictive power of sIgE determinations significantly varies among different food allergens.[71,104,105] There the cutoff values for the same food allergen significantly varied between different studies, where age, demographic, ethnic, and symptomatic dependencies became evident.[31,70,106,107] Hence decision points for sIgE determinations need to be carefully established (1) for each food, (2) for several age groups, and (3) even separately for each health care center.

Specificity of IgE determinations is predominantly affected by homologous cross-reacting proteins, whereas sensitivity mainly depends on the quality of the test agent or other assay limitations. Therefore the use of standardized high-quality allergens has the potential to significantly improve sensitivity and to increase the negative predictive value by decreasing the number of false-negative results (for problems and benefits of sIgE testing with different allergen preparations, see Table I). Accordingly, studies with recombinant allergens revealed substantially improved sensitivity.[16,31,61,88,108,109] It is noteworthy that the use of a mix of

TABLE I. Benefits and problems of allergen preparations used for *in vitro* diagnostics

	Natural extracts	Native allergens	Recombinant proteins
Benefits	Easy to prepare Ideally, all allergenic proteins are present	Enabling of component-resolved diagnosis Native protein structures are mostly preserved Presence of all natural isoforms and posttranslational modifications	Enabling of component-resolved diagnosis and application of a single isoform Lack of impurities with other food proteins Standardization of amount and structural characteristics
Problems	Standardization problems caused by the natural variability of active ingredients (eg, various isoforms with different IgE-binding capacities) and endogenous degradation that also can cause low assay sensitivity Complex mixtures of allergenic and nonallergenic components sometimes resulting in low assay specificity	Laborious preparation Yield depends on composition of source material Risk of variable batch composition caused by different copurification yields of isoforms Risk of low-level contamination with other allergens from the same source and purification artifacts	Laborious preparation Proteins can be unfolded or partially unfolded and might not be properly modified after translation Risk of low-level contamination with components of the expression system and purification artifacts

recombinant major cherry allergens resulted in 95% sensitivity compared with 65% for cherry extract.[31] Supplementation of natural extracts with recombinant allergens is a further promising approach to ensure diagnostic sensitivity and improve quantitative performance.[110] Moreover, component-resolved sIgE determinations with recombinant allergens also promise refined diagnosis. This might be exemplified by the observation that high sIgE titers to recombinant lipid transfer protein in combination with low or absent reactivity to Bet v 1 homologues are clearly correlated with systemic reactions in apple, peach, cherry, and hazelnut allergy, whereas the opposite is true for patients with oral allergy syndrome at consumption of these foods.[31,33,111,112]

Children mostly outgrow class I food allergies.[113] Early RAST studies suggested a correlation of sIgE titers and the pace of tolerance development.[114] Further studies with crude allergen extracts mostly verified a general trend of a correlation between high sIgE titers and slow outgrowth of cow's milk or hen's egg allergy.[115-119] Yet again a significant overlap in serum sIgE reactivity of patients with early and late outgrowth was apparent. However, systematic studies with recombinant food allergens as sensitivity-improved test agents in quantitative sIgE determinations are still lacking. Consequently, the final concept in the diagnosis of food allergy by differentiating quantitative approaches has yet to be defined. Extensive surveys are required using the full set of available recombinant allergens to (1) determine authentic panels of recombinant allergens with respect to best sensitivity performance, (2) define the most reliable cutoff values, and (3) develop reasonable strategies in risk assessment and prediction of permanence.

DIAGNOSIS OF FOOD ALLERGY WITH SYNTHETIC SEQUENTIAL EPITOPES

Today, the stability of class I food allergens is generally believed to be responsible for their capacity to provoke severe systemic reactions.[8] They have the potential to retain IgE reactivity even after digestion, probably because of sequential IgE epitopes, whereas in class II food allergens a single point mutation can result in an almost complete loss of IgE reactivity based on the disruption of the tertiary structure.[10,120-123] Studies on conformational IgE epitopes are rare because the investigation of discontinuous epitopes is far more challenging than the investigation of sequential epitopes. The latter are detectable by means of epitope mapping with captured overlapping synthetic peptides that represent the entire amino acid sequence of the respective allergen.[25] However, this technology has critical limitations. Most importantly, it allows a maximum peptide length of 15 amino acids because of less than 100% efficiency of the coupling reaction.[10] Using 10 to 15 mers at shifting offsets of 1 to 7 amino acids, several patient-specific, as well as immunodominant, epitopes were identified in different class I food allergens.[26,27,29,32,124-130] However, other studies at different shifting offsets, with different peptides sizes, or both revealed different sets of immunodominant sequential epitopes for the same allergens.[29,124-127,130,131]

Nevertheless, approaches to use these alleged immunodominant peptides for the risk assessment of life-threatening symptoms, as well as the prediction of persistence in food hypersensitivity, were apparently successful. In wheat-dependent exercise-induced anaphylaxis, all sera from affected patients showed significant sIgE reactivity to the respective peptides, whereas none of the sera from nonatopic control subjects demonstrated reactivity, and sera from control patients with atopic dermatitis showed very low to nonexistent reactivity.[29,130] The sera of individuals with a history of more severe peanut-induced allergic reactions recognized a higher number of sequential Ara h 1, Ara h 2, and Ara h 3 epitopes than sera from individuals with milder symptoms. However, a higher number of recognized epitopes was also correlated with larger sIgE polyclonality.[132,133] Furthermore, it could be demonstrated that sera of patients with persistent food allergy recognized specific sequential epitopes and showed significantly higher peptide-specific IgE titers than sera of patients who subsequently gained tolerance.[125,134] Individual patterns in sequential epitope binding were variable, and a significant overlap in individual test reactivity between severely affected subjects and patients with milder symptoms was also evident.

In conclusion, risk assessment and prediction of permanence appears to be attainable in class I food allergy by using sequential

epitope-emulating peptides. However, the detection of different immunodominant sequential epitopes in the same allergenic protein, depending on the design of the study, demands definite explanation. Additional high-power studies with quantitative approaches in a larger number of patients are requested to confirm the advantages of sequential peptides with respect to differentiating diagnostics in food allergy.

FUTURE PERSPECTIVES

The use of recombinant food allergens or synthetic sequential epitopes in experimental, as well as commercial, test systems allows, for the fist time, highly sensitive detection of sensitization profiles. IgEs specific to several target allergens, substructures, or both have been suggested as markers of severity, persistence, or both; progress in molecular allergology promises identification of several other targets. Nevertheless, well-designed high-power studies will be required to substantiate current and future findings.

Repeated exposure to cross-reactive antigens increases sIgE polyclonality, as well as the affinity maturation of these antibodies.[135] In cross-sensitized patients this process represents an imminent danger regarding a possible switch from a currently existing pollinosis to a future food-pollen allergy syndrome or from a current single food allergy to multiple food hypersensitivity.[136,137] Regarding a currently not yet clinically relevant cross-reactivity, component-resolved analyses with recombinant allergens promise improved prediction by means of (1) exact quantification of different sIgE titers and (2) quantitative reciprocal cross-inhibition experiments performing sequential preabsorption of the sera with increasing amounts of homologous allergens.[48] Such measurements allow for an estimation of cross-reactive sIgE fractions, as well as the determination of their relative affinities to different homologous cross-reacting proteins. In turn, this enables differentiation between sensitizations with and without the potential for future clinical significance. Consequently, studies with recombinant allergens using modern automated approaches for high-throughput detection of sIgE reactivity might give rise to improved diagnostics.

Microarray technology enables sIgE testing in a multiplex format and allows for the simultaneous measurement of many IgE clones with different specificities in complex arrays comprising large numbers of recombinant allergens, peptide epitopes, or both.[132,138,139] A major advantage lies in the potential to investigate large numbers of analytes in parallel while only using minute amounts of sera and antigens. Therefore, component-resolved analysis with this technology can facilitate simultaneous detection of sIgE abundance, functionality, and interaction concerning numerous allergenic determinants. However, this untargeted "random testing" generates a high amount of not necessarily correlating data. Clinical interpretation would currently rely on available information from testing these reagents by using low-throughout methods with modern bioinformatics approaches to identify novel diagnostic coherences. However, a number of adequately powered clinical trials are required before the introduction of this technology into ordinary clinical practice.

Improved human basophil activation tests use flow cytometric readouts of, for example, CD63 or CD203c for the assessment of activation.[23,24] However, the diagnostic potential of basophil tests with allergen extracts appears not to be superior to sIgE determinations because a broad variability in basophil activity exists between different donors and different allergens.[24] Moreover, an overlap in individual test reactivity between allergic and nonallergic subjects again complicates interpretation.[140] Using recombinant allergens, sequential epitopes, or both might improve the diagnostic reliability, whereas restrictions arising from approval regulations for studies on provocation tests with recombinant allergens are irrelevant. Coupling of the diversity of microarray technology with the assessment of biologic activity in cell-based assays might be used for the parallel detection of several activation markers, enabling reliable estimation of the clinical relevance of sIgE sensitizations at justifiable expenses.[141]

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Updates

The National Heart, Lung, and Blood Institute has made a few changes to the "Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma—Summary Report" since its publication in November 2007 (2007;120:S94-S138). The changes are as follows:

1. Figure 17, "Usual dosages for long-term control medications":
   Under "Oral systemic corticosteroids," "0-4 Years of age": change to read "Short-course burst: 1-2 mg/kg/d, maximum 60 mg/d for 3-10 days" ("60" used to be "30").
2. Figure 19, "Usual dosages for quick-relief medications *(continued)*":
   Under "Systemic corticosteroids," "5-11 Years of age": change to read "Short-course burst: 1-2 mg/kg/d, maximum 60 mg/d, for 3-10 days" (used to be "40-60 mg/d as single or 2 divided doses").
3. Figure 22, "Dosages of drugs for asthma exacerbations *(continued)*":
   Under "Anticholinergics," "Child dose":
   A. "Ipratropium with albuterol," "Nebulizer solution": change to read "1.5-3.0 mL every 20 minutes for 3 doses, then as needed" ("1.5-3.0 mL" used to be "1.5 mL").
   B. "Systemic corticosteroids," "Prednisone": change to read "1-2 mg/kg in 2 divided doses" ("1-2 mg/kg" used to be "1 mg/kg").